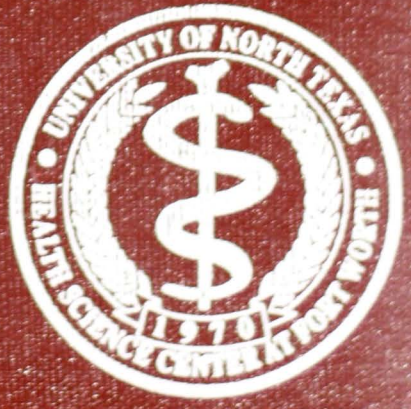
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ABSTRACT

Natural killer (NK) cells are a population of lymphocytes vital for the innate immune response. These cells protect the host during the early phase of infection before the adaptive immune response is effective. NK cells are direct effectors via cytoxicity towards neoplastic and infected cells. Additionally, they modulate the immune response by the production of cytokines, most notably interferon γ and tumor necrosis factor α. Furthermore, NK cell receptors do not undergo rearrangement. Repetoires of activating and inhibitory receptors regulate the functions of NK cells via a balance of signaling. NK cell receptors can broadly be divided by their ligand specificity as well. Most of the known receptors recognize MHC class I molecules and transduce inhibitory signals. This is the basis for the missing self hypothesis espoused by Karre and colleagues. The Ly49 molecules server this function in the mouse and are related to the C-type lectins. In primates, a family of killer inhibitory receptors (KIR) appear to play the same role and are in the immunoglobulin (Ig) superfamily of receptors. Whether humans expressed the Ly49 receptors was a fundamental question in NK cell biology. In my attempt to address this issue, I isolated two receptors related the C-type lectin receptors and localized to the human NK gene complex on chromosome 12 in a region syntenic to where the murine *Ly49* genes reside. Functional characterization of these receptors will facilitate our understanding of NK cell biology.

Additional activating receptors include the members of the CD2 subset of the immunoglobulin superfamily molecules expressed on NK cells and other leukocytes, including murine 2B4. 2B4 is the high affinity ligand for CD48. Engagement of 2B4 on NK cell surfaces with specific antibodies or CD48 can trigger cell mediated cytotoxicity, IFN-γ secretion, phosphoinositol turnover and NK cell invasiveness. This work describes the isolation and characterization of the human homologue of the 2B4 receptor. The putative peptide has a type I structure with one transmembrane domain. The extracellular region is comprised of two immunoglobulin like domains with six putative N-linked glycosylation sites. The cytoplasmic domain of 2B4 contains unique tyrosine motifs (TxYxxV/I) that associate with src homology 2 domain containing protein (SH2D1A) or signaling lymphocyte activation molecule (SLAM)-associated protein (SAP), whose mutation is the underlying genetic defect in the X-linked lymphoproliferative disease (XLPD). Impaired signaling via 2B4 and SLAM is implicated in the immunopathogenesis of XLPD. CS1 is a novel member of the CD2 subset that contains two of the unique tyrosine motifs present in 2B4 and SLAM. Signaling through 2B4, CS1 and other members of the CD2 subset may play a major role in the regulation of NK cells and other leukocyte functions.

MOLECULAR BASIS OF CANCER CELL RECOGNITION AND KILLING BY HUMAN NATURAL KILLER CELLS

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MOLECULAR BASIS OF CANCER CELL RECOGNITION AND KILLING BY HUMAN NATURAL KILLER CELLS

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Kent S. Boles, B.S., M.S.

Fort Worth, Texas

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CHAPTER 1

INTRODUCTION

Lymphocytes include T cells, B cells, and natural killer (NK) cells. Cytotoxic lymphocytes play an important role in destroying damaged and infected cells. The three classes of cytotoxic lymphocytes are αβ cytotoxic T lymphocytes (αβ CTL), γδ T cells, and NK cells. These cells function through the expression of the Fas ligand and release of cytolytic granules. The granules contain perforin and the granzyme family of serine proteases. Additionally, cytolytic lymphocytes play an important role in the innate immune response by releasing cytokines. Most notably, interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) are pivotal in host defense and survival during the early phases of infection. Cytokines regulate immune function by enhancing antigen presentation,, increasing the expression of stimulatory ligands, and by inducing chemotaxis.

NK cells are a population of lymphocytes that play an important role in the innate immune response to certain viruses, bacteria, and parasites (Biron et al., 1999; Scott and Trinchieri, 1995; Unanue, 1997). In humans, NK cells represent 5-20% of lymphocytes circulating in the blood and about 5% of splenic lymphocytes. Alternatively, they are rarely observed in normal lymphatic tissue (Trinchieri, 1989). Human NK cells can be differentiated from other lymphocytes by the expression of CD56 and a lack of CD3. Additionally, they express CD16, the low affinity IgG receptor that allows NK cells to

mediate antibody dependent cellular cytotoxicity (ADCC) (Lanier et al., 1986; Lanier et al., 1983; Perussia et al., 1983). In addition to cellular cytotoxicity, NK cells secrete a variety of cytokines and chemokines. These include IFN-γ, TNF-α, lymphotoxin, IL-3, GM-CSF, IL-5, IL-13, IL-10, IL-8, MIP-1α, MIP1β, lymphotactin, and RANTES (Biron et al., 1999).

The functional relevance of NK cells has demonstrated in a NK cell deficient patient reported in 1989 by Biron et al. (Biron, Byron and Sullivan, 1989). The patient suffered severe, life-threatening infections with the γ herpes viruses, varicella and cytomegalovirus (CMV), but was able to normally control influenza. Other patients lacking NK cells have subsequently been discovered and all have difficulty controlling certain viral infections (Ballas et al., 1990; Joncas et al., 1989). A critical role for NK cells in defense against herpes viruses has been supported by experiment studies in mice lacking NK cells. These animals are unable to control replication of murine cytomegalovirus (Bukowski et al., 1985; Welsh et al., 1991). Furthermore, the natural susceptibility of certain strains of mice to cytomegalovirus infection has been mapped to chromosome 6 in a region termed the NK gene complex due the presence of many NK cell receptor genes (Forbes et al., 1997; Scalzo et al., 1990; Scalzo et al., 1992). Additionally, in experimental models of infection, NK cells have been implicated in resistance against Listeria monocytogenes (Bancroft et al., 1987; Unanue, 1997; Wherry, Schreiber and Unanue, 1991), malaria (Doolan and Hoffman, 1999), Toxoplasma gondii (Denkers et al., 1993; Sher et al., 1993), and Leishmania (Laskay, Rollinghoff and

Solbach, 1993). The ability of NK cells to produce IFN-γ rapidly after infection has been shown to be the protective agent in many of these disease models.

NK cells are distinct from T and B cells in that they do not require RAG genes or a thymus to develop (Spits, Lanier and Phillips, 1995). Regardless, they exhibit a clear capacity to recognize target cells. In the case of tumor cell targets, sensitivity to NK cells was correlated in many instances with decreased levels of MHC expression (Ljunggren and Karre, 1985). NK cells are also able to reject bone marrow grafts, especially in scenarios where the donor graft lacks MHC molecules of the host (Bennett, 1987). These observations led to the formulation of the "missing self" hypothesis by Kärre and colleagues (Karre et al., 1986). It asserts that NK cells ignore potential targets expressing normal levels of autologous class I molecules and attack cells that do not (Figure 1). The missing self hypothesis was sufficient to explain NK cell function *in vivo*, as neoplastic and infected cells often down regulate or lose class I surface expression (Garrido et al., 1997; Tortorella et al., 2000). The molecular mechanism for the recognition of missing self was established when several MHC class I specific receptors were discovered that inhibit NK cell function. It is now apparent that missing self recognition is only one of several types of NK cell target recognition. Stimulatory receptors on NK cells have been identified with ligand specificities for molecules upregulated on tumor cells and stressed cells, and others apparently specific for ligands on normal cells. This variety of recognition systems can function in a model in which NK cells signaling is regulated by the balance of signaling via stimulatory receptors, specific for diverse ligands, and inhibitory receptors, specific for MHC class I molecules.

NK receptors are often arranged in gene clusters due to gene duplication. These families include members that are either activating or inhibitory. These signaling differences arise from variance within the transmembrane and cytoplasmic domains. NK cell receptors belong to either of two superfamilies, the immunoglobulin (Ig) or C-type lectins (Figure 2). Membership in a family is determined by homology. Ig superfamily members contain at least one domain homologous to the constant region of immunoglobulins. The C-type CRD (carbohydrate recognition domain) is the common feature among Ca^{2+}-dependent animal lectins and structurally related proteins. A subset of the C-type lectin family found on NK cells contains domains homologous to other C-type lectin domains, but whether they mediate interactions through carbohydrate or protein binding remains unresolved (Weis, Taylor and Drickamer, 1998).

Figure 1. Diagram depicting the missing self hypothesis. In the presence of activating receptor stimulation, a lack of inhibitory receptor signaling leads to target cell lysis.

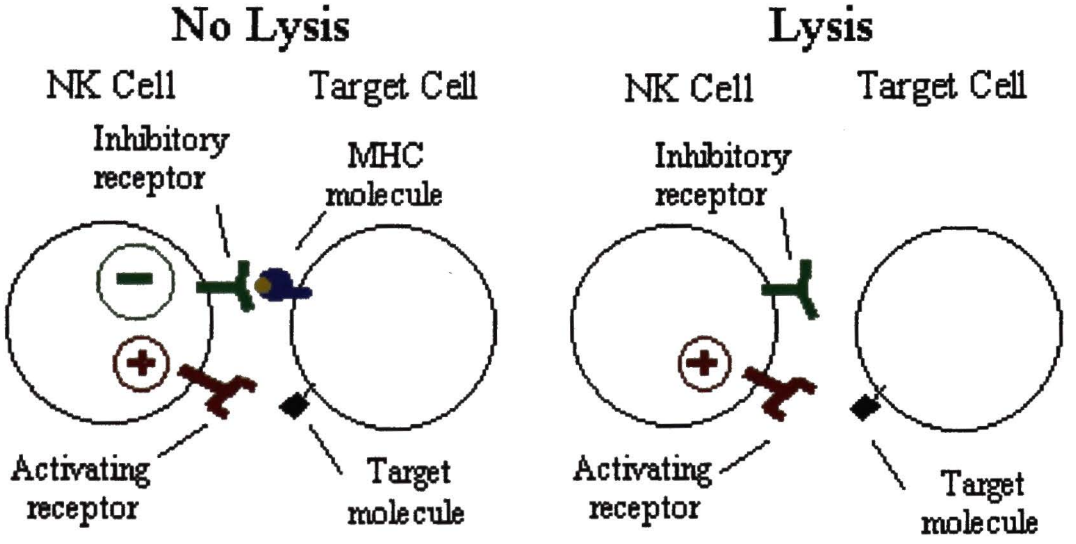
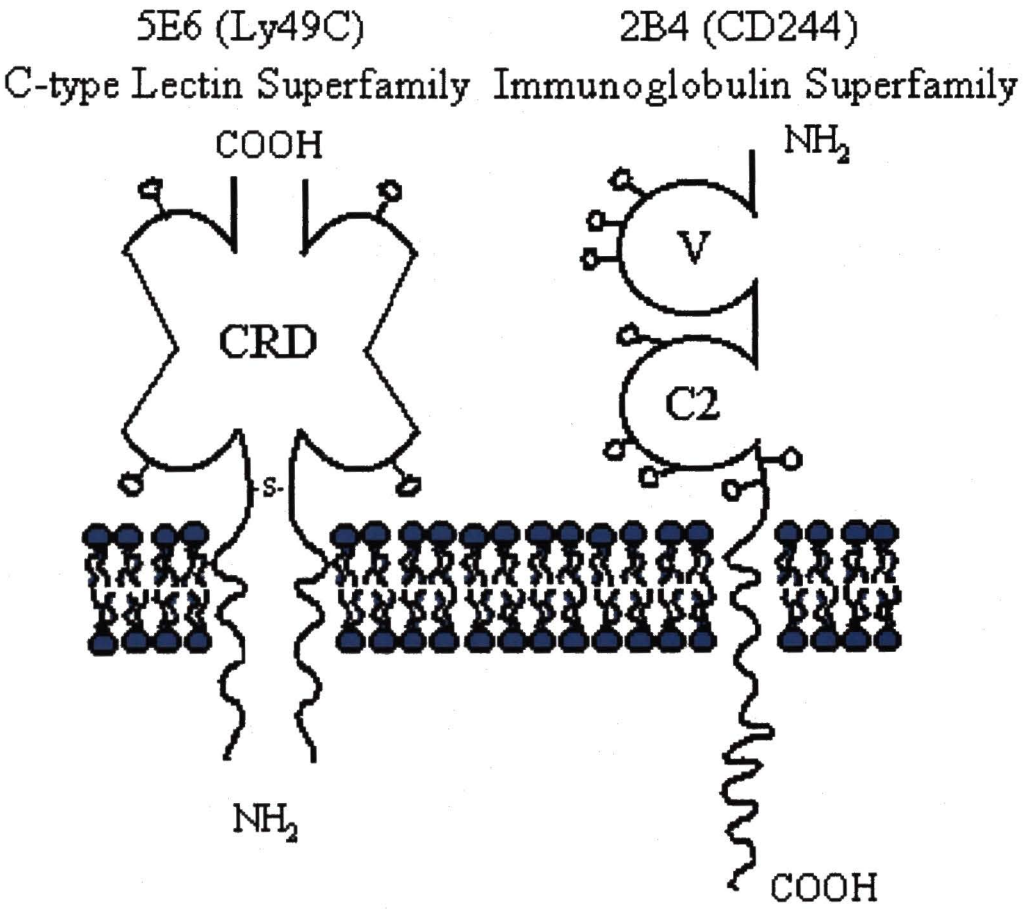

No Lysis
NK Cell
Target Cell
Inhibitory receptor
MHC molecule
Activating receptor
Target molecule
Lysis
NK Cell
Target Cell
Inhibitory receptor
Activating receptor
Target molecule

Figure 2. Representatives of the C-type lectin superfamily and immunoglobulin superfamily, receptors expressed on natural killer cells. CRD represents the conserved carbohydrate recognition domain of the C-type lectins. V and C2 represent domains homologous to respective immunoglobulin domains conserved in the immunoglobulin superfamily.


5E6 (Ly49C)
C-type Lectin Superfamily
2B4 (CD244)
Immunoglobulin Superfamily
COOH
NH2
CRD
V
C2
S-
NH2
COOH

The first inhibitory MHC-specific receptors to be discovered were the Ly49 receptors in rodents (Karlhofer, Ribaudo and Yokoyama, 1992), which bind directly to classical MHC class I molecules (Hanke et al., 1999; Michaelsson et al., 2000; Tormo et al., 1999). More than 12 different Ly49 receptors were identified in mice, though just 8 of these are of the inhibitory type (Smith, Karlhofer and Yokoyama, 1994; Wong et al., 1991a). The second family discovered was the killer cell immunoglobulin-like receptors (KIR) family, which appears to be functional in primates but not rodents, and which also bind directly to class I molecules (Boyington et al., 2000; Long et al., 1997). The third family is functional in both primates and rodents, and it consists of CD94/NKG2 heterodimers (Carretero et al., 1997; Lazetic et al., 1996; Lopez-Botet et al., 1998). Several NKG2 isoforms can pair with the CD94 receptor; only CD94/NKG2A is known to be inhibitory. CD94/NKG2 receptors monitor class I molecules indirectly by specifically recognizing peptides processed from the leader sequences of class I molecules, bound into the groove of a non-classical class I molecule, Qa-1 in mice (Vance et al., 1998) or HLA-E in humans (Borrego et al., 1998; Braud et al., 1998; Lee et al., 1998). Direct and indirect recognition of class I molecules may serve compensatory roles in inhibiting NK cells.

All three of the families of inhibitory receptors signal through motifs in their cytoplasmic domains, called immunoreceptor tyrosine-based inhibitory motifs (ITIM) with the consensus sequence of V/IxYxxL/V (Burshtyn et al., 1997). Upon receptor engagement, ITIM are tyrosine-phosphorylated and recruit protein tyrosine phosphatases such as SHP-1 and possibly SHP-2 (Burshtyn et al., 1996; Campbell et al., 1996; Vely et

al., 1997). Since inhibition is apparent at early activation steps such as calcium mobilization (Leibson, 1997), the phosphatases probably interfere early in NK cell stimulation.

A syntenic region within the genome of both mice and men is termed the NK gene complex. It is on chromosome 6 in the mouse and 12 in the human. NK cell receptors with lectin-like domains are encoded in the NK gene complex and some receptors such as NKPR1, CD94, and the NKG2s are common to both mice and men (Brown et al. 1997; Yabe et al. 1993; Yokoyama and Seaman 1993). A fundamental question in the NK cell receptor field was whether Ly49 molecules expressed in mice would have homologues in the human immune system. Some labs approached the question facilitated by knowledge of the NK cell gene complex in the mouse and the relative positions of the *Ly49* genes. This strategy yielded the identification of only a single non-functional Ly49-like gene in the human (30, 31). Alternatively, I attempted to isolate Ly49 homologues by homology at the RNA transcript level. As chapter 2 illustrates, I initially screened a cDNA library from NK cells with a probe generated from the Ly49C receptor. No likely candidates were observed so I utilized the EST (expressed sequence tagged) database at the National Center for Biotechnology Information (NCBI). I selected several potential receptor sequences that demonstrated homology to the Ly49 receptors and isolated three transcripts. The transcripts are bona fide receptors, but their function and relation to the Ly49s remains in question.

For many years, the characterization of stimulatory receptors on NK cells lagged behind that of inhibitory NK receptors. The possibility was suggested that adhesion

molecules alone, such as LFA-1, might be responsible for the required NK cell activation signal (Lanier, Corliss and Phillips, 1997). However, subsequent evidence suggests several stimulatory receptors in NK cell target recognition. Activating receptors on NK cells can be broadly divided into those that recognize MHC class I like ligands and those that do not. Examples of receptors ligands in the latter include the NKG2D receptor's ligands MICA/B, Rae1, and H60 (Bauer et al., 1999; Cerwenka et al., 2000; Diefenbach et al., 2000). ADCC is mediated when CD16, an FcR recognizes cell bound IgG. Lastly, CD48 is the high affinity ligand for 2B4 (Brown et al., 1998; Latchman, McKay and Reiser, 1998). Interestingly, if interactions between MHC molecules and receptors on NK cells are prevented, NK cells will attack normal autologous lymphoblasts (Ciccone et al., 1994; Hoglund et al., 1991; Liao et al., 1991), a fact suggesting that some stimulatory receptors on NK cells react with non-MHC ligands expressed by normal cells. Candidate receptors for such reactions include NKp30, NKp44, and NKp46 receptors, as well as the NK1.1 antigen (NKR-P1C) (Moretta et al., 2001). Additionally, MHC class I specific stimulatory receptors are found within each of the three families of class I specific receptors. The KIR contain the stimulatory KIR2DS and KIR3DS, the NKG2 family contains the stimulatory NKG2C and NKG2E receptors (Vance, Jamieson and Raulet, 1999), and the Ly49 family contains stimulatory Ly49D and Ly49H receptors (Mason et al., 1996; Smith et al., 1998). Expression of these stimulatory receptors overlaps with expression of class I specific inhibitory receptors. A beneficial consequence of this expression pattern is that a target cell that has lost an inhibitory class I allele while retaining a stimulatory class I allele would become highly sensitive to attack.

NK cell stimulatory receptors generally associate with small transmembrane adapter proteins that transmit activation signals, including KARAP/DAP12 (Lanier et al., 1998; Olcese et al., 1997), CD3ζ , FcεRIγ (Wirthmueller et al., 1992), and DAP10/KAP10 (Chang et al., 1999; Wu et al., 1999). It is thought that each of these adapters is expressed by all NK cells but associates only with a distinct subset of the stimulatory receptors. These adapters contain an immunoreceptor tyrosine-based activation motif (ITAM) and consequently transduces stimulatory signals via association with Src family kinases (Gaul et al., 2000; Visco et al., 2000). The consensus sequence for the ITAM is I/VxYxxL/Vx_{26-31}I/VxYxxL/V (Tomlinson, Lin and Weiss, 2000; Weiss and Littman, 1994). NK cells from mice or humans with mutations in one of these genes, KARAP/DAP12, exhibit only a subtle phenotype and can still attack most NK target cells (Bakker et al., 2000a; Tomasello et al., 2000c). These data confirm the idea that NK cell activation is multifactorial and does not rely on a single signaling adapter.

Members of the CD2 subset of receptors play a major role in lymphocyte functions, do not recognize MHC molecules, and have not been shown to interact with ITAM containing adaptor molecules. Signaling lymphocyte activation molecule, SLAM (CD150), a member of the CD2 subset, is expressed on T cells and B cells and regulates T cell activation and production of Ig by B cells (Cocks et al., 1995; Sidorenko and Clark, 1993). 2B4 (CD244) is expressed on NK cells and other leukocytes (Mathew et al., 1993). It was originally identified as an activation receptor on mouse NK cells and T cells that are involved in non-MHC-restricted killing (Garni-Wagner et al., 1993; Mathew et al., 1993). Identification of CD48 as the high affinity ligand of 2B4 implicated a broader

role of 2B4 in immune regulation (Brown et al., 1998; Latchman, McKay and Reiser, 1998). Isolation of human 2B4 has allowed exploration of the role of 2B4 in human immune disease (Boles et al., 1999b; Tangye et al., 1999). Recently, a wealth of information has emerged from several laboratories on the various functional roles of 2B4 in immune regulation. In X-linked lymphoproliferative disease, NK cells cannot be activated via surface 2B4 (Benoit et al., 2000; Nakajima et al., 2000; Parolini et al., 2000; Tangye et al., 2000). The molecular adaptor protein, SAP/SH2D1A (SLAM-associated protein or SH2 domain containing adaptor molecule) associates with the cytoplasmic tail of 2B4 and SLAM (Sayos et al., 2000; Tangye et al., 1999). Defective signaling via 2B4 and SLAM may contribute to the pathogenesis of X-linked lymphoproliferative disease due to mutations in SAP. 2B4 contains four novel tyrosine motifs (TxYxxV/I) in the cytoplasmic domain (Boles et al., 1999b; Mathew et al., 1993). A close relative of 2B4, SLAM, also contains these novel tyrosine motifs. The signaling mechanism of 2B4 is still unclear.

Chapter 3 describes the cloning and initial characterization of the human 2B4 receptor. More recently, I identified another member of the CD2 subset, CS1 that may regulate NK cell function as described in chapter 4. The presence of two of the unique tyrosine motifs in the cytoplasmic domain of CS1 suggests that it may associate with SAP and thus regulate immune responses. *2B4* and *CS1* genes are localized to human chromosome 1 along with other members of the CD2 subset. In chapter 5 I describe the structural and functional aspects of 2B4 and CS1 in light of the role of other members of CD2 subset in immune regulation.

CHAPTER 2

IDENTIFICATION OF NOVEL C-TYPE LECTIN RECEPTORS ON HUMAN NK CELLS

Introduction

NK cell receptors with lectin-like domains are encoded in the NK gene complex on chromosome 6 in the mouse and 12 in the human (Brown et al., 1997; Yabe et al., 1993; Yokoyama and Seaman, 1993). The majority of NK cell receptors encoded by the NK gene complex belong to groups of highly related genes such as the *NKR-P1, Ly-49*, and *NKG2* families. The Ly-49 and NKG2 families contain members that are mostly inhibitory, but have a few members that transduce activation signals (Lanier, 1998a; Long and Wagtmann, 1997; Yokoyama and Seaman, 1993). The NKR-P1 receptors have been observed to act as activating receptors in rodents. Cross-linking of the human NKR-P1 homologue with antibody leads to inconsistent results (Lanier, Chang and Phillips, 1994; Poggi et al., 1996) suggesting the possibility of isoforms that have not been isolated as of yet (Lanier, 1998a). CD94 is a type II receptor expressed on most NK cells and was originally implicated as an inhibitory receptor (Chang et al., 1995; Long and Wagtmann, 1997). Subsequently, it was discovered to form a hetero-dimer with members of the NKG2 family (Lazetic et al., 1996).

CD69 and AICL (activation induced C-type lectin) are two structurally similar receptors localized to the NK gene complex, but have interesting differences to the other

genes located there. As opposed to the other type II receptors in the NK gene complex, which are restricted to NK cells and a subset of T cells, CD69 and AICL are expressed in most cells of hematopoietic origin (Hamann et al., 1997; Lanier, 1998b; Long and Wagtmann, 1997; Testi et al., 1994). The function of AICL is not known, but CD69 cross-linking leads to the activation of NK cells, T cells, B cells, monocytes, granulocytes, and platelets (Testi et al., 1994). Additionally, these genes appear to have single rather than multiple isoforms.

My goal in isolating new lectin-like receptors was two-fold. First I sought to generally characterize the mechanism by which NK cells recognize their targets by isolating any potential signal transduction molecules. Secondly, I attempted to isolate any Ly49 homologues that might be expressed in the human as opposed to the murine system. Screening a cDNA library with the Ly49C cDNA was unsuccessful and I have omitted the results from this dissertation. Alternatively, I searched the EST database and was able to generate several potential candidates for NK cell receptors with lectin-like homology.

Methods

EST database search and cDNA library screening

The EST (expressed sequence tag) database at NCBI (http://www.ncbi.nlm.nih.gov) was searched with the TblastN program versus a consensus sequence of human (CD69, CD94, and NKG2s) and mouse (Ly-49s) C-type lectin receptors (Boguski, 1995; Boguski, Lowe and Tolstoshev, 1993). Several overlapping clones were identified and PCR primers were designed to amplify a fragment

within the C-type lectin domains of candidate transcripts (Table 1). cDNA from a NK cell library constructed in λ phage by Dr. J. Houchins (R & D Systems, Minneapolis, MN and kindly provided by Dr. A. Brooks, NIH, Bethesda, MD) was successfully used as template. PCR cycle conditions were 94° C for 30 seconds, 50° C annealing temperature for 30 seconds, and a 72° C extension for 45 seconds repeated for 30 cycles using *Taq* DNA polymerase from GIBCO BRL (Grand Island, NY) at 2 mM $MgCl_2$. The same library was then screened with the resulting PCR fragments. Each was labeled with α-^{32}P dCTP (Feinberg and Vogelstein, 1983; Sambrook, Fritch and Maniatis, 1989). Approximately, $5x10^5$ clones were screened per candidate. After three rounds of screening, phage DNA was isolated from positive clones by the method of Lee et al. (Lee and Clark, 1997b). All positively selected clones were sequenced (Automated sequencing facility, Department of Pathology, UT Southwestern Medical Center, Dallas) and analyzed (Genetics Computer Group, Wisconsin package). Three clones which contained open reading frames were identified for further study. The transcripts were named LLT1-3 (lectin-like transcript 1-3) due to sequence similarity to other C-type lectin-like receptors found on NK cells.

Table 1. PCR primers used to amplify LLT and CD69 sequences.

name	gene	sequence (5'-3')	sense
YF1	LLT1	GAGCTAACTGCCATCAAGAGCCATCAGTATGTC	+
YR1	LLT1	AGAACCTAAGTAAGCTCCTTGAACACTTTGGAA	-
RBC141	LLT1	TTCCTGTTGAGATATAAAGGC	+
RBC136	LLT1	GGAACAAATCCACTTCCTCT	-
EST1F1	LLT2	GCA CGA GGC TTT ACT CAC	+
EST1R1	LLT2	CTG AAA CTC CCA AGT CTA ACC	-
RBC151	CD69	GGGTCTGACAAGTGTGTTTT	+
RBC130	CD69	TGCCACATCACATATTGCAC	-
β actin F	β actin	TAC CAC TGG CAT CGT GAT GGA CT	+
β actin R	β actin	TCC TTC TGC ATC CTG TCG GCA AT	-

Cell culture

Human tumor cell lines Jurkat (T cell), YT (NK cell), HL-60 (monocytic), and DB (B cell), in addition to a murine lymphoma cell line (YAC-1), were cultured in RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml of penicillin and streptomycin, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Gibco BRL, Grand Island, NY). A lymphokine activated killer cell (LAK) culture was obtained by isolating peripheral blood mononuclear cells (PBMC) from 60 ml of venous blood from a healthy donor by Ficol-Paque centrifugation (Pharmacia, Piscataway, NJ). The cells were grown in the above media supplemented with 1000 U /

ml of human rIL-2 for three days. The non-adherent cells were removed and the culture was continued in 500 U / ml of human rIL-2 and conditioned media until day 10, when RNA was extracted. All cell lines were grown to one million per ml and split 1:2 twenty-four hours before RNA isolation.

RNA and DNA blot analysis

Total RNA was isolated with the RNAstat 60 reagent according to the manufacturer's protocol (Teltest Inc., Friendswood, TX), divided into 20 μg aliquots, and stored in 70% EtOH at -80° C until used. 1% agarose gels for Northern analysis were stained with Ethidium bromide after electrophoresis to insure equal loading by comparison of rRNA. Northern blots were probed with 25 ng of the full length cDNA labeled with α^{32}P dCTP (Feinberg and Vogelstein, 1983; Sambrook, Fritch and Maniatis, 1989). The first blot (Figure 6A) consisted of 20 μg of total RNA from human monocytic, T, B, and NK cell lines (HL-60, Jurkat, DB, and YT, respectively), a mouse cell line (YAC-1), and LAK and PBMC cells from a healthy donor immobilized on Hybond nylon (Amersham, Arlington Heights, IL). Prehybridization and hybridizations were performed per the instructions of Amersham for the Hybond nylon membrane at 65° C. The second membrane was purchased from Clontech (Palo Alto, CA) and contained mRNA samples from human spleen, lymph node, thymus, peripheral blood leukocytes, bone marrow and fetal liver (Human Immune System Multiple Tissue Northern Blot II). It was hybridized per the manufacturer's directions with the included ExpressHyb Hybridization solution at 65° C (Figure 6B). Blots were exposed to Hyperfilm (Amersham, Arlington Heights, IL).

The membrane was subsequently stripped and reprobed for β actin to insure equal loading.

Genomic DNA was isolated from human liver by a standard protocol (Sambrook, Fritch and Maniatis, 1989). For DNA blot analysis, human genomic DNA samples (20 μg each) were digested with various restriction enzymes (BamH I, EcoR I, Hind III, and Xba I) and separated on 0.8% agarose gel by electrophoresis. The DNA was transferred to Hybond nylon membrane under alkaline condition (0.4N NaOH) and fixed by UV cross linking. The membrane was prehybridized for 2 hours at 65^{0}C in hybridization buffer (1 mM EDTA, 0.5 M sodium phosphate, pH 7.2, 7% SDS, 100 mg/ml ssDNA). The probe (50 ng of the full length LLT1 cDNA labeled with α^{32}P dCTP) was added to the same buffer and hybridizations continued for 18 hr at 65° C (Feinberg and Vogelstein, 1983; Sambrook, Fritch and Maniatis, 1989). The membrane was washed with a buffer containing 40 mM sodium phosphate, pH 7.2, 1% SDS at 65^0 C for 1 hr. The filter was exposed to Hyperfilm for one day (Amersham, Arlington Heights, IL) and developed (Figure 8A). Subsequently, the membrane was stripped by immersion in boiling 0.5% SDS and reprobed with the LLT2 cDNA as above (Figure 8B).

RT-PCR analysis of transcript expression

Twenty μg of total RNA from each of the indicated cell lines was utilized in a 50 μl reverse transcription reaction. Superscript II was used per the manufacturer's directions (Invitrogen). Approximately, 200 ng of each cDNA sample was utilized in a PCR reaction with the LLT2 specific primers indicated in Table 1. Each reaction was in a

volume of 50 μl and utilized the RedTaq polymerase and buffer (Sigma). All of each reaction was loaded on a 1% agarose gel and visualized by ethidium bromide staining (Figure 7). Furthermore, similar reactions for β actin were performed to insure the integrity of the cDNA.

Results

Isolation and sequence analysis of the LLT cDNA clones

The molecular basis of target cell recognition by NK cells is poorly understood. Unlike T and B cells, NK cells do not rearrange DNA to generate diversity. Therefore, one could predict that NK cells might express several receptors to recognize various targets or utilize some other mechanism to generate diversity. In fact, over the last few years a number of receptors have been identified on NK cells (Lanier, 1998b). However, all the functions of NK cells could not be accounted by the known receptors. In order to understand the mechanism by which NK cells recognize and kill target cells, I searched the EST database with a consensus sequence of human (CD69, CD94, and NKG2s) and mouse (Ly-49s) C-type receptors (Boguski, 1995; Boguski, Lowe and Tolstoshev, 1993). Several overlapping clones were identified and PCR primers were designed and used in PCR to yield a fragment within the C-type lectin domain. The primers used for PCR amplifications are given in Table 1. We screened a human NK cell cDNA library with the PCR fragment and a positive clone were selected for further analysis.

The first clone (LLT1) contained a cDNA insert of 950 bp with an open reading frame predicting a polypeptide of 181 amino acid residues with a type II receptor

structure (Genbank accession number AF133299). The predicted protein sequence had a single transmembrane domain of 19 amino acid residues (Figure 3A and B) and an intracellular domain of 30 amino acid residues. Additionally, it had an extracellular lectin-like domain of 132 amino acid residues which contained two putative N-linked glycosylation sites (Figure 3A).

Analysis of the sequences of LLT1 clones from another NK cell cDNA library made from pooled NK cells (NKTRP, kindly provided by Dr. Brent Passer, NIH, Bethesda, MD) revealed no differences indicating that the gene is not highly polymorphic. Furthermore, sequence data available in the EST database did not show variation beyond what was expected for single-pass sequences.

Figure 3. Analysis of the cDNA sequence of LLT1. A) The nucleotide sequence and predicted translation of LLT1. The transmembrane domain is underlined. Glycosylation sites in the extracellular domain are boxed. B) Hydrophilicity plot of the LLT1 putative peptide sequence determined by the Kyte-Doolittle method. This figure was reproduced from Kent S.Boles, Roland Barten, Pappanaicken R. Kumaresan, John Trowsdale, and Porunelloor A. Mathew. 1999. Cloning of a new lectin-like receptor expressed on human NK cells. *Immunogenetics* 50: 1-7 with permission.

A

```
AAT TCC GGC AAA ATG CAT GAC AGT AAC AAT GTG GAG AAA GAC ATT ACA CCA TCT GAA TTG CCT GCA AAC CCA GGT TGT CTG CAT TCA AAA   91
                M   H   D   S   N   N   V   E   K   D   I   T   P   S   E   L   P   A   N   P   G   C   L   H   S   K    26

GAG CAT TCT ATT AAA GCT ACC TTA ATT TGG CGC TTA TTT TTC TTA ATC ATG TTT CTG ACA ATC ATA GTG TGT GGA ATG GTT GCT GCT TTA   181
 E   H   S   I   K   A   T   L   I   W   R   L   F   F   L   I   M   F   L   T   I   I   V   C   G   M   V   A   A   L    56

AGC GCA ATA AGA GCT AAC TGC CAT CAA GAG CCA TCA GTA TGT CTT CAA GCT GCA TGC CCA GAA AGC TGG ATT GGT TTT CAA AGA AAG TGT   271
 S   A   I   R   A   N   C   H   Q   E   P   S   V   C   L   Q   A   A   C   P   E   S   W   I   G   F   Q   R   K   C    86

TTC TAT TTT TCT GAT GAC ACC AAG AAC TGG ACA TCA AGT CAG AGG TTT TGT GAC TCA CAA GAT GCT GAT CTT GCT CAG GTT GAA AGC TTC   361
 F   Y   F   S   D   D   T   K   N   W   T   S   S   Q   R   F   C   D   S   Q   D   A   D   L   A   Q   V   E   S   F   106

CAG GAA CTG AAT TTC CTG TTG AGA TAT AAA GGC CCA TCT GAT CAC TGG ATT GGG CTG AGC AGA GAA CAA GGC CAA CCA TGG AAA TGG ATA   451
 Q   E   L   N   F   L   L   R   Y   K   G   P   S   D   H   W   I   G   L   S   R   E   Q   G   Q   P   W   K   W   I   136

AAT GGT ACT GAA TGG ACA AGA CAG TTT CCT ATC CTG GGA GCA GGA GAG TGT GCC TAT TTG AAT GAC AAA GGT GCC AGT AGT GCC AGG CAC   541
 N   G   T   E   W   T   R   Q   F   P   I   L   G   A   G   E   C   A   Y   L   N   D   K   G   A   S   S   A   R   H   166

TAC ACA GAG AGG AAG TGG ATT TGT TCC AAA TCA GAT ATA CAT GTC TAG ATG TTA CAG CAA AGC CCC AAC TAA TCT TTA GAA GCA TAT TGG   631
 Y   T   E   R   K   W   I   C   S   K   S   D   I   H   V                                                              181

AAC TGA TAA CTC CAT TTT AAA ATG AGC AAA GAA TTT ATT TCT TAT ACC AAC AGG TAT ATG AAA ATA TGC TCA ATA TCA CTA ATA ACT GGG   721
AAA ATA CAA ATC AAA ATC ATA GTA AAA TAT TAC CTG TTT TCA TGG TGC TAA TAT TAC CTG TTC TCC CAC TGC TAA TGA CAT ACC CGA GAA   811
TGA GTA ATT TAT AAA TAA AAG AGA TTT AAT TGA AAA AAA                                                                    950
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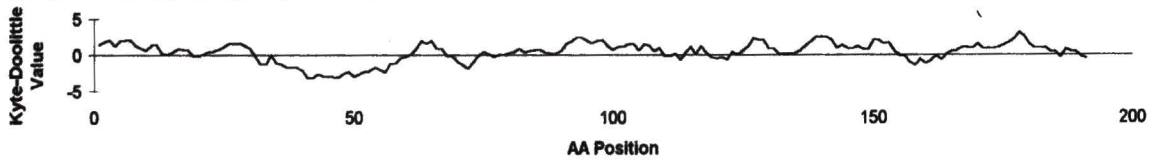
B



The LLT2 clone contained a cDNA insert of 1542 bp with an open reading frame predicting a polypeptide of 242 amino acid residues with a type II receptor structure. The predicted protein sequence had a single transmembrane domain of 31 amino acid residues (Figure. 4A and B) and an intracellular domain of 12 amino acid residues. Additionally, it had an extracellular lectin-like domain of 199 amino acid residues which contained two putative N-linked glycosylation sites (Figure 4A). The predicted protein sequence of LLT1 has an extracellular domain with homology to the C-type lectin-like domains shared with other NK cell receptors (Weis, Taylor and Drickamer, 1998). It has the highest similarities to AICL and CD69 of 59 and 56%, respectively (Hamann, Fiebig and Strauss, 1993; Hamann et al., 1997; Lopez-Cabrera et al., 1993; Ziegler et al., 1993). Additionally, representative homologies to other NK cell receptors belonging to the C-type lectin superfamily are 53, 51, and 41% to NKG2-D, CD94, and Ly-49D, respectively (Chang et al., 1995; Houchins et al., 1991; Weis, Taylor and Drickamer, 1998; Wong et al., 1991b). LLT2 had 55 and 47% similarity compared to LLT1 (Figure 5).

Figure 4. Analysis of the cDNA sequence of LLT2. A) The nucleotide sequence and predicted translation of LLT2. The transmembrane domain is underlined. Glycosylation sites in the extracellular domain are boxed. B) Hydrophilicity plot of the LLT2 putative peptide sequence determined by the Kyte-Doolittle method. This figure was reproduced from Kent S. Boles, Roland Barten, Pappanaicken R. Kumaresan, John Trowsdale, and Porunelloor A. Mathew. 1999. Cloning of a new lectin-like receptor expressed on human NK cells. *Immunogenetics* 50: 1-7 with permission.

A

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GAA TTC GGC ACG AGG CTT TAC TCA CCG CAC TCA GGA CGC TGG CTT CTC TGT TGT TTC TCA ACC ATG TCA GGC GTG CTT CCA CCT CAA GAG   90
                                                                M   S   G   V   L   P   P   Q   E                      9

CAC AGG GCT CCC TCT TCA ACG TGG CGA CCA GTG GCC CTG ACC CTG CTG ACT TTG TGC TTG GTG CTG CTG ATA GGG CTG GCA GCC CTG GGG  180
 H   R   A   P   S   S   T   W   R   P   V   A   L   T   L   L   T   L   C   L   V   L   L   I   G   L   A   A   L   G   39

CTT TTG TTT TTT CAG TAC TAC CAG CTC TCC AAT ACT GGT CAA GAC ACC ATT TCT CAA ATG GAA GAA AGA TTA GGA AAT ACG TCC CAA GAG  270
 L   L   F   F   Q   Y   Y   Q   L   S   N   T   G   Q   D   T   I   S   Q   M   E   E   R   L   G   N   T   S   Q   E   69

TTG CAA TCT CTT CAA GTC CAG AAT ATA AAG CTT GCA GGA AGT CTG CAG CAT GTG GCT GAA AAA CTC TGT CGT GAG CTG TAT AAC AAA GCT  360
 L   Q   S   L   Q   V   Q   N   I   K   L   A   G   S   L   Q   H   V   A   E   K   L   C   R   E   L   Y   N   K   A   99

GGA GCA CAC AGG TGC AGC CCT TGT ACA GAA CAA TGG AAA TGG CAT GGA GAC AAT TGC TAC CAG TTC TAT AAA GAC AGC AAA AGT TGG GAG  450
 G   A   H   R   C   S   P   C   T   E   Q   W   K   W   H   G   D   N   C   Y   Q   F   Y   K   D   S   K   S   W   E   129

GAC TGT AAA TAT TTC TGC CTT AGT GAA AAC TCT ACC ATG CTG AAG ATA AAC AAA CAA GAA GAC CTG GAA TTT GCC GCG TCT CAG AGC TAC  540
 D   C   K   Y   F   C   L   S   E   N   S   T   M   L   K   I   N   K   Q   E   D   L   E   F   A   A   S   Q   S   Y   159

TCT GAG TTT TTC TAC TCT TAT TGG ACA GGG CTT TTG CGC CCT GAC AGT GGC AAG GCC TGG CTG TGG ATG GAT GGA ACC CCT TTC ACT TCT  630
 S   E   F   F   Y   S   Y   W   T   G   L   L   R   P   D   S   G   K   A   W   L   W   M   D   G   T   P   F   T   S   189

GAA CTG GTC CAT ATT ATA ATA GAT GTC ACC AGC CCA AGA AGC AGA GAC TGT GTG GCC ATC CTT AAT GGG ATG ATC TTC TCA AAG GAC TGC  720
 E   L   V   H   I   I   I   D   V   T   S   P   R   S   R   D   C   V   A   I   L   N   G   M   I   F   S   K   D   C   219

AAA GAA TTG AAG CGT TGT GTC TGT GAA AAA AGG GCA GGA ATG GTG AGC CCA aAG AGC TCC ATG TCC CCC TGA ACA TTA GGC GAA GAC AGA  810
 K   E   L   K   R   C   V   C   E   K   R   A   G   M   V   S   P   K   S   S   M   S   P                          242

GGA TCT CAA GGC ATA CTT CTC ACA GAT AAA ATA CTT CCA CGG TGC TGC AGT TAG ACG AAA GAA GGG AAT TGC TTT TCA NGT ATC CAC AGA  900
CTT GGT TAG CTG ACT GGG ATC TCT CTG CTG GCA ACC TGG AAA AGC AAG TCA ACA TTT CTG AGG TTA GAC TTG GGA GTT TCA GAA ATG CTC  990
CTG AGG GTA ACT AAT GAG AGG TGT GTA GCT GCT TGT TTT GTT TTG TGT TGA CAC AGC AAC TTC CTG TGG TGG TTT CAT CCT CCT TCT TCA 1080
GTA ACT GCT TCT TTC TCT CAA TAA AGA ATG TTA ATA TAA TTT GAA CAA AAA CAG CAT GTA AAG AAA AAG AAA ACA TAG ATA CCC AAA GAC 1170
AGT CCC TAA ATT AGA ATC TCT TTG CTG ACT CTT AAT TTT CAA AAT ATG AAG GCA CCT GAT TCT GCT TCT CCA TCT CCC CAC TGC CAT NTG 1260
AAA AGC TTA ATG TGG AAA TAA ATA GTT ATT CTC TAA ACA TTA AGC GAT ACA AAA GAA ACA CAC TTG ATA ATT ATA TTA TTC ATC AGA CAC 1350
TCA AAT TGT GAG GTG AAT TTT CTA TAA GAT AAT AGA AGC AGG GCT GGG AAG GAG AGA GAC TTA GAA GGC ATG AAG CTA CTC TTA ATT TTG 1440
TTA GCT TTA ATC CCA ATT AGC CGC CTG TAA CAT TAT CAT GCT GCT TGT CCC TTG GAG AAA ATT CAT GGT ACA AGG TGA AAT AAA CTT TTG 1530
AAT TCC AAA AAA                                                                                                      1542
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B

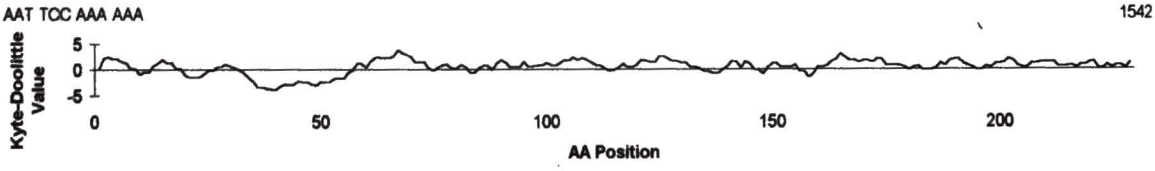


Figure 5. Alignment of amino acid sequences the LLT receptors and other c-type lectin receptors. Conserved residues are shaded. Glycosylation sites in the extracellular domain are boxed. Transmembrane domains are underlined. The amino acid comparison was compiled by using the PILEUP program in the Genetics Computer Group software. This figure was reproduced from Kent S. Boles, Roland Barten, Pappanaicken R. Kumaresan, John Trowsdale, and Porunelloor A. Mathew. 1999. Cloning of a new lectin-like receptor expressed on human NK cells. *Immunogenetics* 50: 1-7 with permission.


LLT1
LLT2
AICL
CD69
NKG2-D

Expression of LLT1 in different tissues and cells

The expression of LLT1 transcripts in various cell lines and different human tissues was analyzed by Northern blotting of total RNA or poly(A)$^+$ RNA. The full length cDNA hybridized to transcripts of approximately 5, 3.5, 2, and 0.9 kb in total RNA from a human NK cell line (YT) and hybridized weakly to transcripts of similar sizes from human T cell (Jurkat), B cell (DB), or monocytic (HL-60) tumor cell lines (Figure 6A). Hybridization signals for the same size transcripts were strong in donor samples from a LAK culture and PBMC except for the 900 bp transcript (Figure 6A). Tissue distribution of LLT1 showed that human peripheral blood leukocytes, lymph node, thymus, and spleen expressed transcripts of the same relative sizes as the YT cell line with the exception of the 900 bp transcript (Figure 6B). No hybridizing transcripts were detected in mRNA from fetal liver or bone marrow. LLT1 may be expressed only in the later stages of NK cell differentiation, similar to Ly49 expression.

Expression of LLT2 in different tissues and cells

The expression of LLT2 transcripts in various cell lines was analyzed by RT-PCR of total RNA. The LLT2 primers (Table 1) amplified a specific 350 bp band from a human NK cell line (YT), B cell (DB), or monocytic (HL-60) tumor cell lines (Figure 7). No PCR fragments were amplified in human T cell (Jurkat) or PBMC from a health donor. Alternatively, cDNA from a LAK culture (same donor as the PBMC) contained the transcript suggesting that LLT2 might be inducible.

Figure 6. RNA blot analysis of LLT1 transcripts hybridized with 32P labeled, full length LLT1 cDNA. A) Total RNA (20 μg) isolated from the YAC-1, HL-60, DB, Jurkat, and YT tumor cell lines was electrophoresed in a formaldehyde agarose gel, blotted and probed. Additionally, samples were included from the PBMC and a LAK culture from a healthy donor. B) Northern blot of poly (A)+ RNA from spleen, lymph node, thymus, peripheral blood leukocytes, bone marrow, and fetal liver tissues. Both membranes were stripped and hybridized with a β-actin probe. The position of 28S and 18S rRNA and the sizes of RNA molecular standards are shown at the left and right of panels A and B, respectively. This figure was reproduced from Kent S. Boles, Roland Barten, Pappanaicken R. Kumaresan, John Trowsdale, and Porunelloor A. Mathew. 1999. Cloning of a new lectin-like receptor expressed on human NK cells. *Immunogenetics* 50: 1-7 with permission.

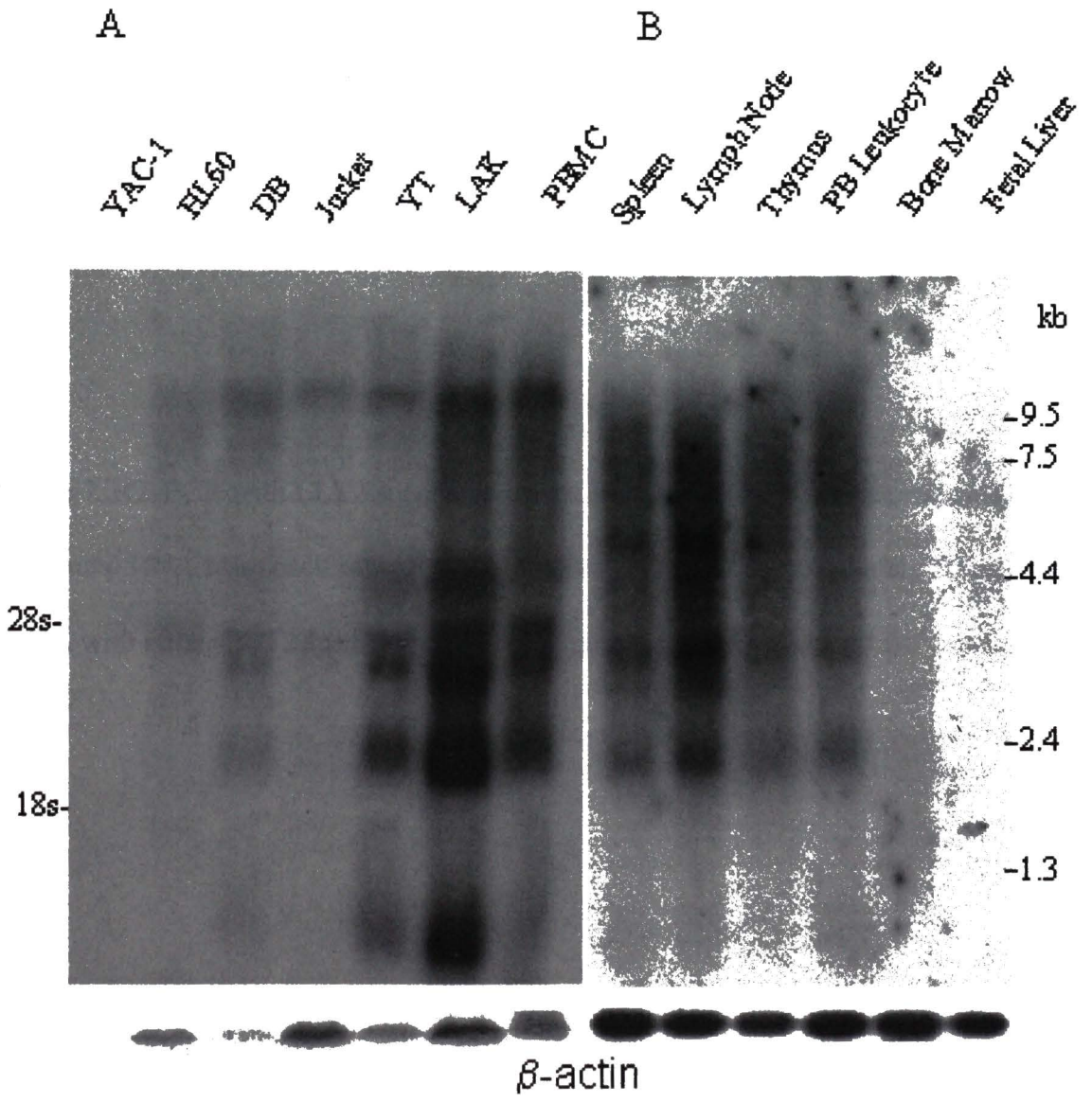
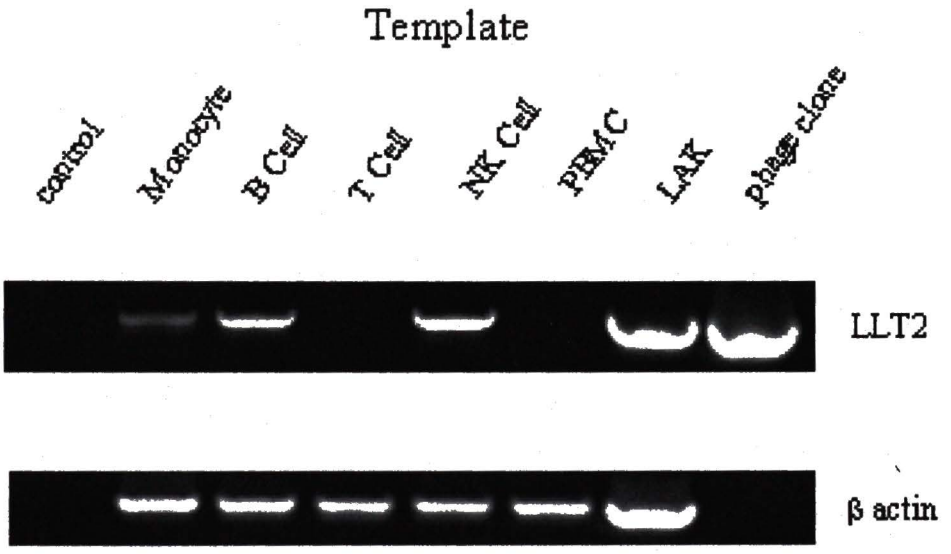

A
B
YAC-1
HL60
DB
Jurkat
YT
LAK
PBMC
Spleen
Lymph Node
Thymus
PB Leukocyte
Bone Marrow
Fetal Liver
kb
9.5
7.5
4.4
2.4
1.3
28s-
18s-
β-actin

Figure 7. RT-PCR with LLT2 specific primers. First strand cDNA was produced from 20 μg of total RNA from each sample. PCR was performed on approximately 50 ng of each cDNA with either LLT2 specific primers or β actin primers.


Template
control
Monocyte
B Cell
T Cell
NK Cell
PBMC
LAK
phage clone
LLT2
β actin

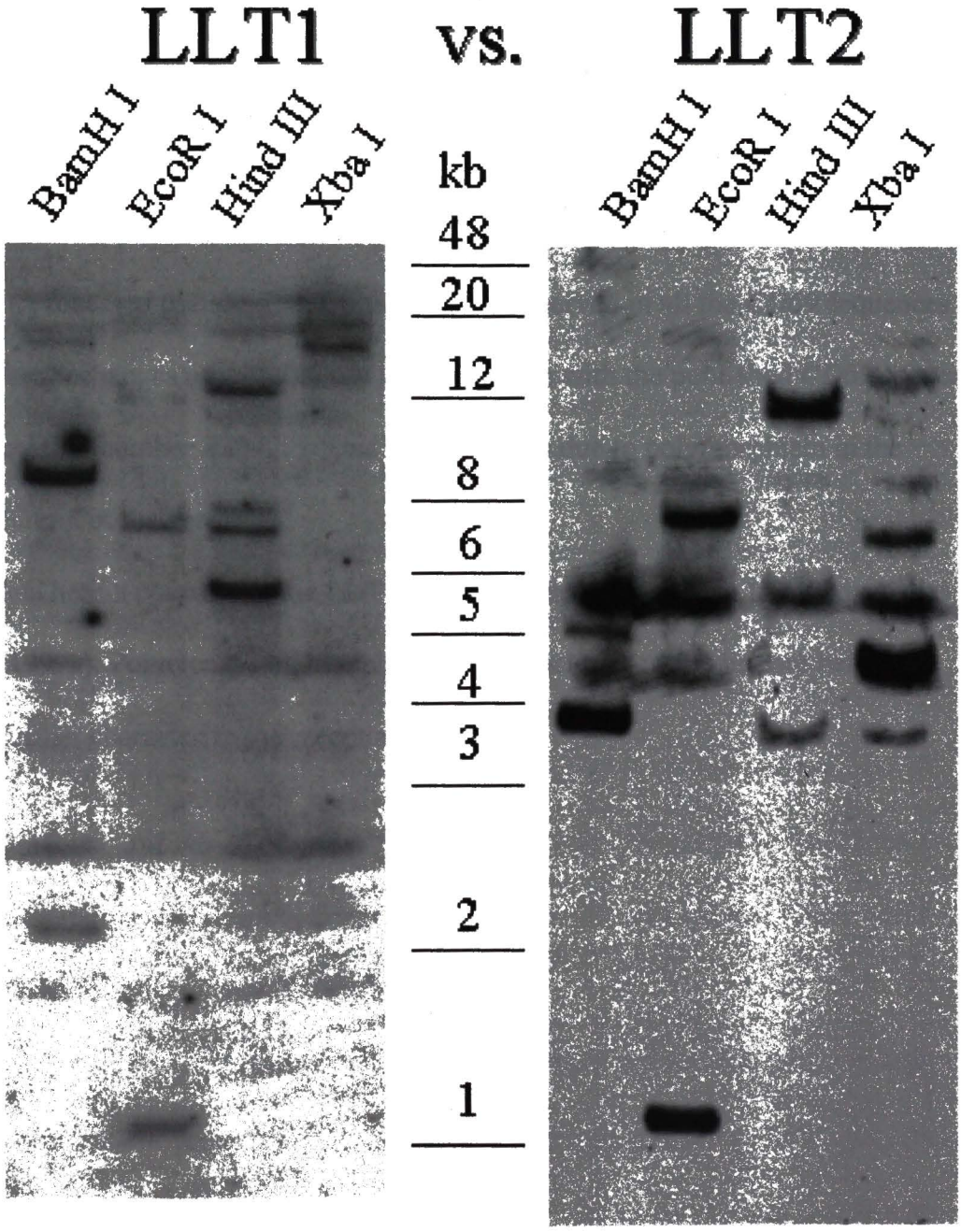
Southern analysis of human genomic DNA

Several lectin-like receptors expressed on NK cells belong to multigene families (Lanier, 1998a; Long and Wagtmann, 1997; Weis, Taylor and Drickamer, 1998). Southern blot analysis of human genomic DNA was carried out to explore this possibility for LLT1. Genomic DNA was isolated from human liver and digested with four different restriction enzymes (BamH I, EcoR I, Hind III, and Xba I), separated on an agarose gel and transferred to a nylon membrane. The full length LLT1 cDNA hybridized to several restriction fragments (Figure 8). The strongly hybridizing restriction fragments identified in Figure 8 ranged from 22 to 35 kb for the different digestions in addition to several weakly hybridizing bands. The membrane was stripped and reprobed with the LLT2 cDNA. A similar, but different pattern of hybridization was observed.

Chromosomal localization

Due to the sequence similarity of the LLT1 clone to AICL and CD69, I expected that *LLT1* might be localized in the NK gene complex on chromosome 12. Therefore, a PAC library containing the NK gene complex was screened. Two PACs were isolated from the human RCP1 library using a probe for LLT1. The inserts of PAC NKCP4 and NKCP5 were sized on a pulsed field gel to 110 and 160 kb, respectively.

Figure 8. Genomic DNA blot analysis of the LLTs. Aliquots of human genomic DNA (20 μg) from liver were digested with the restriction enzymes BamH I, EcoR I, Hind III, and Xba I, electrophoresed in 0.8% agarose, blotted, and hybridized with a 32P labeled, full length LLT1 or LLT2 cDNA. Sizes of DNA standards are shown at the left. Portions of this figure was reproduced from Kent S. Boles, Roland Barten, Pappanaicken R. Kumaresan, John Trowsdale, and Porunelloor A. Mathew. 1999. Cloning of a new lectin-like receptor expressed on human NK cells. *Immunogenetics* 50: 1-7 with permission.


LLT1
vs.
LLT2
BamH I
EcoR I
Hind III
Xba I
kb
48
20
12
8
6
5
4
3
2
1
BamH I
EcoR I
Hind III
Xba I

LLT1 is located in the human NK gene complex within 100 kb of the *CD69* gene. PCR products of approximately 0.9 kb (RBC150/RBC130) as expected for the CD69 gene and 1.8 kb (RBC136/RBC141) for the *LLT1* gene were obtained with both PAC DNA's as templates. The PCR products were sequenced. The *LLT1* specific PCR product sequence revealed amplification of an intron. The exon sequence showed 100% identity to the cDNA of LLT1. The presence of intron sequence is consistent with this being the authentic *LLT1* gene and not a processed pseudogene. Consistently, I observed in all the *CD69* sequences three nucleotide exchanges (out of 796 bp) in comparison with the published *CD69* sequence (Genbank accession number Z30428). This is most likely due to polymorphism in the untranslated 3' end of the gene.

The data herein concerning the LLT1 transcript was published in Immunogenetics (Boles et al., 1999a). Polyclonal sera in rabbits and monoclonal antibodies are being produced to further characterize the receptor.

CHAPTER 3

CLONING AND CHARACTERIZATION OF THE HUMAN HOMOLOGUE OF THE 2B4 RECEPTOR

Introduction

In order to identify cell surface receptors involved in the recognition and activation of NK cells by target cells, a panel of monoclonal antibodies against purified NK cells was developed in the laboratory of Michael Bennet and Vinay Kumar. NK cells from C57BL/6 mice were used to immunize 129 Sv/J mice (Sentman et al., 1989). One antibody, previously designated, SW2B4 (mAb2B4) stained virtually all NK cells and some T cells from C57BL/6 but not 129, BALB/c, DBA/2, or AKR/J mice (Garni-Wagner et al., 1993). Furthermore, SW2B4 activated both cytolytic function and IFN-γ production of lymphokine (IL-2) activated killer (LAK) cells. Treatment of LAK cells with SW2B4 mAb induced killing of a variety of tumor target cells (RMA, RMA-S and P815 but not K562), and induced IFN-γ production.

Splenic T cells cultured in high concentrations of IL-2 acquire the ability to kill NK-sensitive tumor cells. When this population of T cells was sorted into $2B4^+$ and $2B4^-$ cell populations, all of the YAC-1 (prototypic murine NK cell target) killing was found in the $2B4^+$ population. Treatment of these cells with anti-2B4 mAb increased killing of YAC-1 target cells. Additionally, murine dendritic epidermal T cells (DETC) are skin

tissue resident γδ T cells and express 2B4. Freshly isolated DETC express low levels of 2B4, but when cultured in IL-2, expression of 2B4 is greatly increased. The increased 2B4 expression is correlated with increased cytolytic activity against YAC-1 cells, (Schuhmachers et al., 1995a). Further studies of 2B4 function on DETC (Schuhmachers et al., 1995b) demonstrated that, as with LAK cells, culture of some DETC lines with soluble SW2B4 mAb rapidly induced IFN-γ production. In addition, plate-bound SW2B4 (but not soluble) mAb stimulated proliferation and IL-2 production with some DETC lines. Additionally, co-stimulation by plate-bound SW2B4 mAb was observed when DETC cells were sub-optimally stimulated with plate-bound anti-CD3 mAb, suggesting a 2B4 dependent co-stimulation signal.

The SW2B4 antibody was utilized to expression clone the cDNA for 2B4 (Mathew et al., 1993). Analysis reveals that the 2B4 receptor is a member of the Ig superfamily. Furthermore, it belongs to the CD2 subfamily which also includes CD2, CD48, CD58, CD84, CDw150 (SLAM), and Ly9. Both SLAM and 2B4 appear to produce several splice variants from the same gene (Punnonen et al., 1997; Stepp et al., 1999b). SLAM makes four different transcripts including a cytoplasmic, a soluble, and two membrane forms. Interestingly, the difference in the membrane forms is due to splicing within the cytoplasmic tail; this leads to the loss of the two distal of the four tyrosine motifs in the longer form to produce the shorter form. The original 2B4 clone reported contained four tyrosine motifs in the cytoplasmic tail. Recently, a short splice variant was reported which is produced by alternative splicing of the 2B4 transcript (Stepp et al., 1999b). The extracellular domains of murine short and long isoforms are

identical while the shorter form is missing the two distal tyrosines at the C terminal. Thus, the murine 2B4 follows the same splicing pattern as that seen in SLAM molecules. When long and short 2B4 cDNA's were expressed in a cytolytic rat NK tumor cell line (RNK-16), they inhibited or stimulated lytic activity in redirected lysis assays, respectively (Schatzle et al., 1999). Thus, it appears that the short and long isoforms of 2B4 have opposing functions.

Many NK cell receptors bind MHC class I molecules as their ligands (Lanier, 1998b; Long and Wagtmann, 1997). In contrast, CD2 subfamily members bind homophilically to members of the CD2 subfamily. CD2 binds CD58 in the human and CD48 in the rodent (Arulanandam et al., 1993; Kato et al., 1992; Sandrin et al., 1993; van der Merwe et al., 1994; van der Merwe et al., 1993). Additionally, SLAM is a self ligand leading to activation of lymphocytes (T and B cells) (Punnonen et al., 1997). Recently, CD48 was observed to be the high affinity ligand for both murine and human 2B4 (Brown et al., 1998; Latchman, McKay and Reiser, 1998). CD48 is widely expressed in leukocytes and its soluble form is detectable in the blood (Smith et al., 1997). This is especially provocative because CD48 gene knockout mice appear to have immunodeficiencies whereas the CD2 knockout mice appear to be normal (Killeen, Stuart and Littman, 1992).

The responsiveness of murine NK cells to 2B4 stimulation led me to isolate the human homologue of the receptor. The possibility that it may mediate similar activation of human NK cells yields potential for the use of NK cells in the treatment of various diseases such as autoimmune disorders and the immunotherapy of cancer. The murine

cDNA failed to hybridize to human RNA samples in northern analysis (Figure 9A). Alternatively, it did hybridize to EcoR I digested mouse and human genomic DNA fragments (Figure 9B). Therefore, I isolated a genomic fragment (Figure 10A and B) which potentially contained some of the *human 2B4* gene. Indeed, a putative coding sequence had 70% homology with the murine cDNA and did hybridize to human RNA samples (Figure 9C). I subsequently utilized the genomic fragment to isolate the cDNA for the human 2B4 receptor. This chapter describes the cloning and initial characterization of the receptor.

Figure 9. Probe generation strategy for the cloning of the human 2B4 cDNA. A) Northern blot of mouse and human total RNA versus a full length mouse cDNA for 2B4. The mouse sample was from the CTLL-2 cell line, a murine T cell lymphoma. The human sample was total RNA isolated from the peripheral blood mononuclear cells of a healthy donor. B) Genomic Southern analysis of mouse and human DNA versus a full length mouse cDNA for 2B4. C) Northern blot of mouse and human total RNA versus a human genomic DNA fragment with homology to the cytoplasmic domain of murine 2B4.

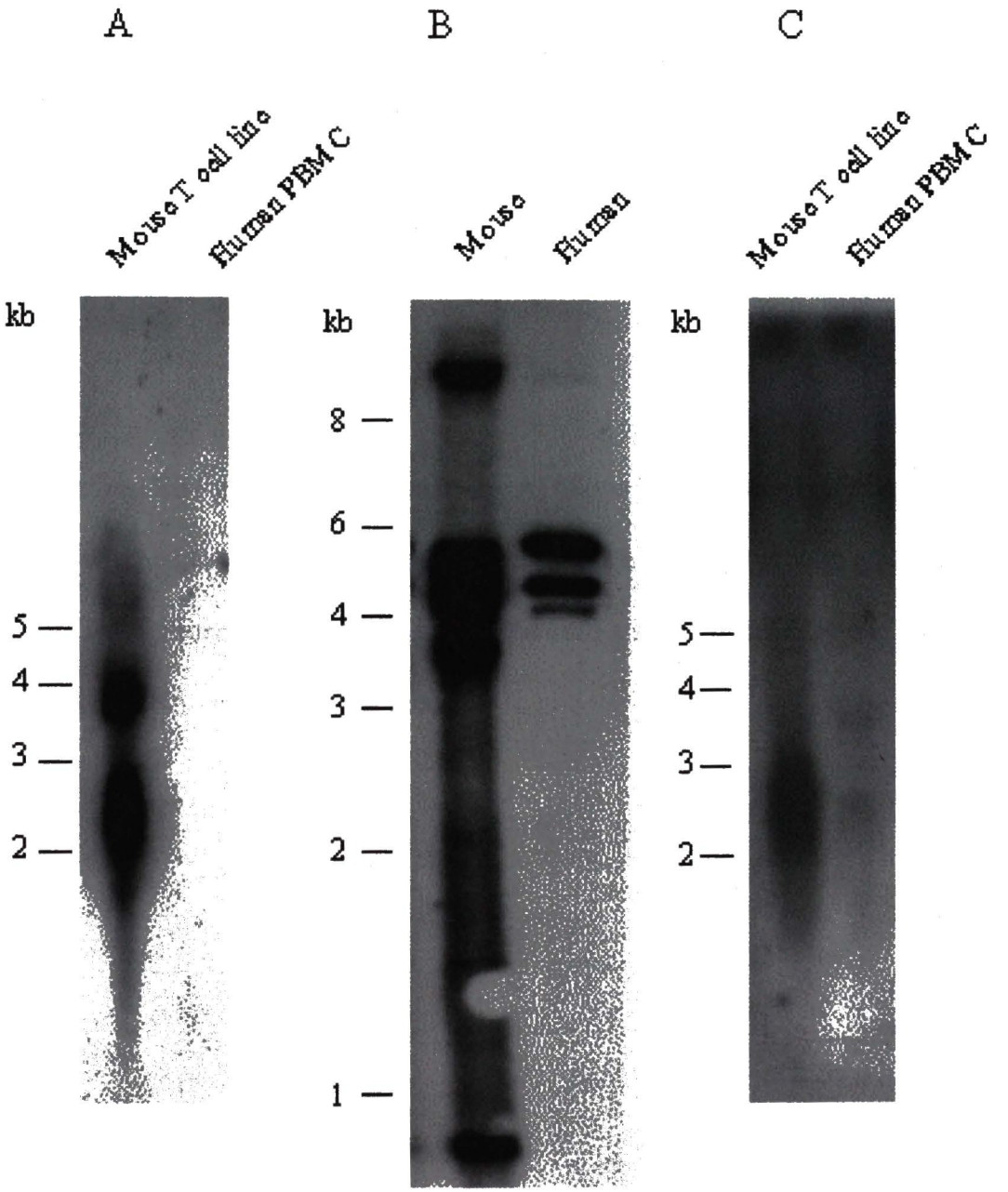
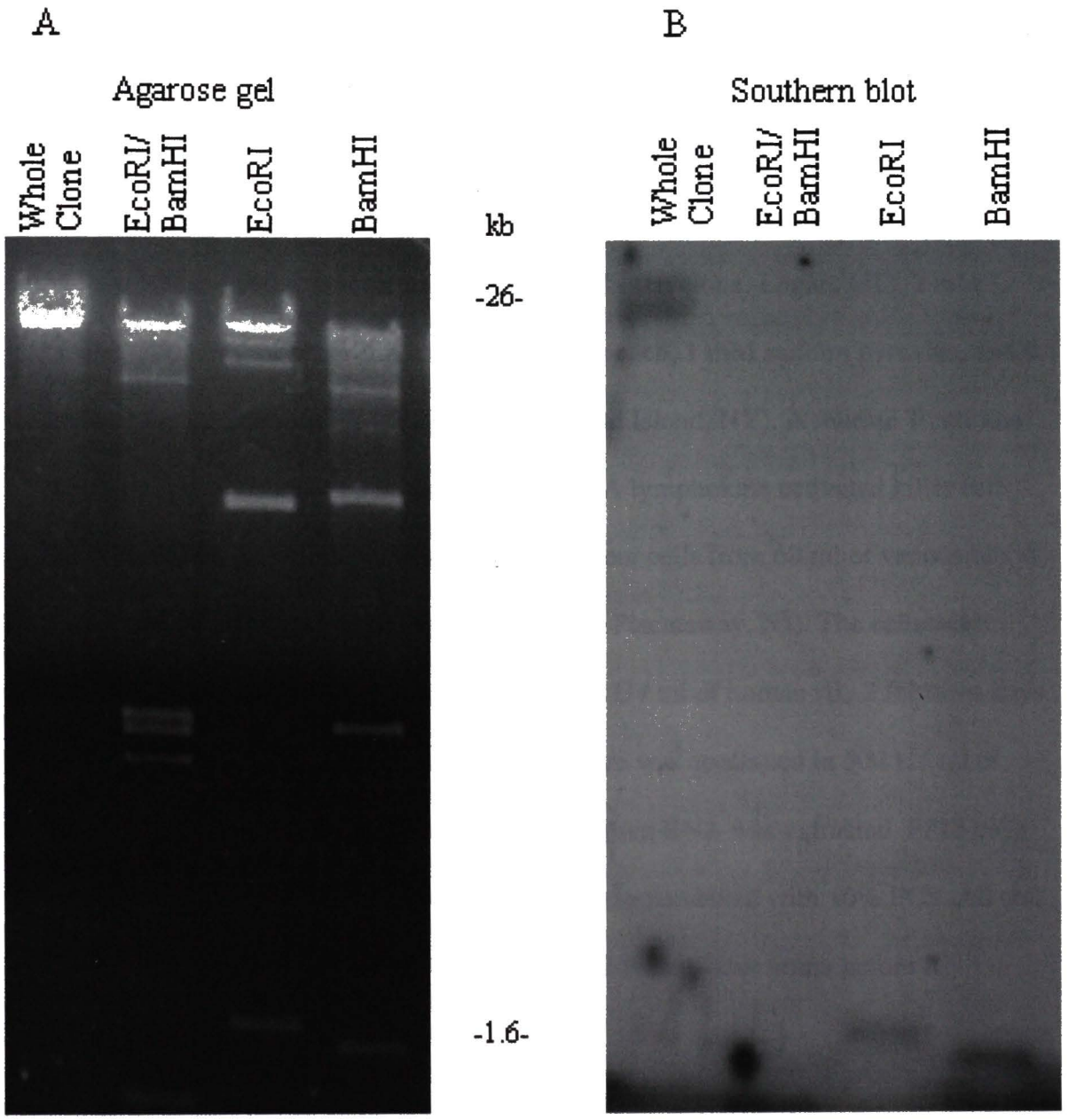

A
B
C
Mouse T cell line
Human PBMC
Mouse
Human
Mouse T cell line
Human PBMC
kb
kb
kb
8
6
5
4
4
3
3
2
2
1
5
4
3
2

Figure 10. Human 2B4 genomic clone analysis. A) Clone H7A1A was digest with various restriction endonucleases and separated on a 0.8% agarose gel with ethidium bromide. B) The agarose gel was Southern blotted onto a nylon membrane and hybridized to a full length cDNA probe from the mouse.


A
Agarose gel
Whole Clone
EcoRI/ BamHI
EcoRI
BamHI
kb
-26-
-1.6-
B
Southern blot
Whole Clone
EcoRI/ BamHI
EcoRI
BamHI

Methods

Tissue culture

Human tumor cell lines Jurkat (T cell), K562 (erythroleukemia cell), YT (NK cell line) and 721.221 (MHC class I deficient EBV-transformed B cell line) cells were cultured in RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml of penicillin and streptomycin each, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (GIBCO BRL, Grand Island, NY). A murine T cell line (CTLL-2) was also maintained in the same media. A lymphokine activated killer cell (LAK) culture was obtained by isolating mononuclear cells from 60 ml of venous blood from a healthy donor with Ficol Paque (Pharmacia, Piscataway, NJ). The cells were grown in the above media supplemented with 1000 U / ml of human rIL-2 for three days. The non-adherent cells were removed and the culture was continued in 500 U / ml of human rIL-2 and conditioned media until day 12, when RNA was extracted. P815 cells (mouse mastocytoma cell) were grown in DMEM supplemented with 10% FCS. All cell lines were grown to one million per ml and split 1:2 twenty-four hours before RNA isolation.

RNA and DNA blot analysis

Total RNA was isolated with the RNAstat 60 reagent according to the manufacturer's protocol (Teltest Inc., Friendswood, TX), divided into 20 μg aliquots, and stored in 70% ethanol at -80° C until used. Northern blots were probed with 25 ng of the full length cDNAs or the human genomic fragment labeled with $\alpha^{32}P$. The blots consisted of 20 μg of total RNA from cell lines or donor samples immobilized on Hybond nylon membrane (Amersham, Arlington Heights, IL). Prehybridization and hybridizations were performed per the instructions of Amersham for the Hybond nylon membrane at 65° C. Ethidium bromide staining of 28S and 18S RNA was done to verify that each lane contained equal amounts of RNA (data not shown). The initial northerns contained samples of CTLL-2 and human PBMC total RNA and were probed with either the murine 2B4 cDNA (Figure 9A) or the human genomic fragment (Figure 9C).

Following isolation of the human 2B4 cDNA, additional northern analysis was conducted. The first membrane (Figure 13A) consisted of total RNA from a T cell and NK cell line (Jurkat and YT, respectively). Additionally samples from a LAK culture and a CD56 subtracted LAK culture were included. The cell sorting was accomplished with Dynal anti-CD56 paramagnetic beads using the manufacturer's protocol. The second membrane (Figure 13B) was purchased from Clontech (Palo Alto, CA) and contained equal amounts of mRNA samples from human spleen, lymph node, thymus, peripheral blood leukocytes, bone marrow and fetal liver (Human Immune System Multiple Tissue Northern Blot II). It was hybridized per the manufacturer's directions with the included ExpressHyb Hybridization solution at 65° C. Blots were exposed to Hyperfilm

(Amersham, Arlington Heights, IL) for two weeks. Subsequently, the membrane was stripped and reprobed with a 2 kb β-actin probe provided by Clontech to ensure equal loading of the mRNA samples.

Genomic DNA was isolated from mouse and human liver by a standard protocol (Sambrook, Fritch and Maniatis, 1989). A Southern blot for cloning was performed with genomic DNA from human and mouse hybridized to the murine cDNA (Figure 9B). Additional DNA blot analysis to characterize the human gene consisted of human genomic DNA samples (20 μg each) digested with various restriction enzymes (BamH I, EcoR I, Hind III, and Xba I) and separated on 0.8% agarose gel by electrophoresis. The DNA was transferred to Hybond nylon membrane under alkaline condition (0.4 N NaOH) and fixed by UV cross linking. The membrane was prehybridized for 2 hours at 65^0 C in prehybridization buffer (1 mM EDTA, 0.5 M sodium phosphate, pH 7.2, 7% SDS, 100 μg/ml ssDNA). The probe (50 ng of the full length cDNA labeled with α^{32}P dCTP) was added to the same buffer and hybridizations continued for 12 to 18 hr at 65^0 C. The membrane was washed with a buffer containing 40 mM sodium phosphate, pH 7.2, 1% SDS at 65^0 C for 1 hr. The filter was exposed to Hyperfilm for one day (Amersham, Arlington Heights, IL) and developed (Figure 12).

Genomic clone isolation

A human genomic library purchased from Stratagene (La Jolla, CA) was screened to isolate exonic DNA sequences that could act as better probes than the murine cDNA of 2B4. Approximately $5x10^5$ clones were screened by plaque lift with Hybond nylon

membrane (Amersham, Arlington Heights, IL). The murine cDNA (25 ng) was labeled with α^{32}P dCTP by random priming method of Feinberg and Vogelstein (Feinberg and Vogelstein, 1983) using the mega-prime labeling kit (Amersham, Arlington Heights, IL). Hybridization was performed per the instructions of the manufacturer for the Hybond nylon membrane at 65° C. Phage DNA was isolated from individual clones by a method recently described by Lee and Clark (Lee and Clark, 1997a) and sequenced through the services of the Automated Sequencing Facility, Dept. of Pathology, UT Southwestern Medical Center, Dallas. Sequence data was analyzed with various programs in the GCG package (Genetic Computer Group, Wisconsin). The clones were identical containing approximately 20 kb inserts. One μg of insert was digested with either BamH I or EcoR I (Figure 10A). The murine cDNA (25 ng, α^{32}P labeled) hybridized to only one 1.6 kb band in the EcoR I digest in a Southern blot (Figure 10B). A similar digest and gel were run and the same band was cloned into the sequencing vector pGemscript I . Sequence analysis revealed a 180 bp region with 70% similarity to a murine cDNA region corresponding to the cytoplasmic tail of murine 2B4. PCR primers (EF1, 5'-AAGAGTCAACCTAAAGCCCAGAA-3'; ER1, 5'-TATAGAGACTCCTGTGCCGTCAT -3') were designed to amplify the 180 bp fragment. Cycle conditions were 94° C for 30 seconds, 50° C annealing temperature for 30 seconds, and a 72° C extension for 45 seconds repeated for 30 cycles using *Taq* DNA polymerase from GIBCO BRL (Grand Island, NY).

cDNA library screening

A human NK cell cDNA library constructed by Dr. J. Houchins (R & D Systems, Minneapolis, MN and kindly provide by Dr. A. Brooks, NIH) was screened using the 180 bp genomic fragment labeled with $\alpha^{32}P$. Approximately $5x10^5$ clones were screened for three rounds and positive phage DNA was isolated (Lee and Clark, 1997a). All clones were sequenced by automated sequencing (Automated Sequencing Facility, UT Southwestern Medical Center, Dallas). One full length clone was identified for further study. The cDNA insert from the phage was PCR amplified using SP6 and T7 primer sites located on the pGEM vector. PCR products were cloned into the pCR2.1 vector with the TA cloning kit (Invitrogen, Carlsbad, CA). All clones were completely sequenced.

Assay for lytic activity

Activated human NK cells were tested for the ability to lyse K562, P815, and 221 target cells in a 4 hour ^{51}Cr release assay as described previously (Tutt et al., 1986). The effector cells were incubated for 30 minutes with a control or specific Ab in 96 well, round bottom plates prior to the addition of ^{51}Cr labeled target cells. A mAb generated against human 2B4 was added as a specific Ab. Plates were subsequently incubated at 37° C for four hours. The plates were spun at 250 x g for 7 minutes to pellet the cells and 100 µl of the supernatant was removed and counted by scintillation counting. Percent cytotoxicity was calculated as follows: 100 x (experimental release – spontaneous release) / (maximum release – spontaneous release). Spontaneous release was the measure of radioactivity in the supernatant from target cells without effector cells.

Maximum release was calculated in a similar fashion except the target cells were fully lysed by the addition of 1% NP-40 detergent.

Results

Isolation and partial characterization of a human 2B4 genomic clone

Previous data demonstrated that full length mouse cDNA hybridized to human genomic DNA (Mathew et al., 1993). However, the mouse cDNA did not hybridize to human total RNA from NK cells. This could be due to low expression level of the 2B4 transcript. Therefore, I decided to isolate human exonic fragments for use as high specificity probes. Towards this goal, I screened a human genomic library (Stratagene, La Jolla, CA) and isolated three identical 20 kb clones. In order to identify an exonic region of the genomic clones, I digested them with EcoR I and hybridized with the mouse cDNA (Figure 10A). A 1.6 kb restriction fragment hybridized to the mouse cDNA (Figure 10B) and was isolated and the complete nucleotide sequence was determined. Comparison of the sequence with mouse cDNA revealed a 180 bp region with 70% similarity to the cytoplasmic tail of murine 2B4 long form (Mathew et al., 1993). This region was PCR amplified for use as a human 2B4 specific probe. Isolation and sequence analysis of a human 2B4 cDNA clone

The genomic fragment of human 2B4 was used to screen a cDNA library constructed by J. Houchins (R & D Biosystems, Minneapolis, MN) and kindly provided by A. Brooks (NIH, Bethesda, MD). Four positive clones were isolated. Sequence analysis showed that three of the clones were partial whereas one clone (λh2B4.19a1) was full length. This clone contained a cDNA insert of 1464 bp with an open reading

frame encoding a polypeptide of 365 amino acid residues (Figure 11A). The predicted protein sequence has a leader sequence of 18 amino acids and a single transmembrane domain of 24 amino acid residues (Figure 11A and B). Additionally, it has an extracellular domain of 204 amino acid residues which contains eight putative N-linked glycosylation sites. The cytoplasmic tail is 120 amino acids long with six tyrosine residues. The human receptor has a 33 amino acid deletion between the first and second tyrosines proximal to the membrane as compared to the murine 2B4 long form.

The predicted protein sequence showed 70% similarity to the long form of murine 2B4 and 61% to the short form. Additionally, it has 48, 45, and 43% similarity to human CD84, SLAM, and CD48, respectively (Figure 12). The two Ig-like domains are conserved between these receptors in addition to several tyrosine containing motifs that may function in signaling.

Figure 11. Nucleotide sequence of h2B4 cDNA and the predicted amino acid sequence. *A*, The nucleotide sequence and predicted translation of human 2B4 (Genbank accession AF107761). The signal peptide is underlined. The transmembrane domain is double underlined. Glycosylation sites in the extracellular domain are boxed. Tyrosine containing motifs in the intracellular domain are shaded. *B*, Hydrophilicity plot of the human 2B4 putative peptide sequence determined by the Kyte-Doolittle method. This figure was reproduced from Kent S. Boles, Marco Colonna, Hideo Nakajima, Samuel S. Chuang, Susan E. Stepp, Michael Bennett, Vinay Kumar, and Porunelloor A. Mathew. 1999. Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4. *Tissue Antigens* 54: 27-34 with permission.

A

```
CAG TTC TGC TCC CCA TCG TCC TCT TGC TGA CTG GGG ACT GCT GAG CCC GTG CAC GGC AGA GAG TCT GGT GGGGGGGGAGGGGCTG GCC TGG    90
CCC CTC TGT CCT GTG GAA ATG CTG GGG CAA GTG GTC ACC CTC ATA CTC CTC CTG CTC CTC AAG GTG TAT CAG GGC AAA GGA TGC CAG GGA   180
                M  L  G  Q  V  V  T  L  I  L  L  L  L  L  K  V  Y  Q  G  K  G  C  Q  G                                25

TCA GCT GAC CAT GTG GTT AGC ATC TCG GGA GTG CCT CTT CAG TTA CAA CCA AAC AGC ATA CAG ACG AAG GTT GAC AGC ATT GCA TGG AAG   270
 S  A  D  H  V  V  S  I  S  G  V  P  L  Q  L  Q  P  N  S  I  Q  T  K  V  D  S  I  A  W  K                               55

AAG TTG CTG CCC TCA CAA AAT GGA TTT CAT CAC ATA TTG AAG TGG GAG AAT GGC TCT TTG CCT TCC AAT ACT TCC AAT GAT AGA TTC AGT   360
 K  L  L  P  S  Q  N  G  F  H  H  I  L  K  W  E  N  G  S  L  P  S  N  T  S  N  D  R  F  S                               85

TTT ATA GTC AAG AAC TTG AGT CTT CTC ATC AAG GCA GCT CAG CAG CAG GAC AGT GGC CTC TAC TGC CTG GAG GTC ACC AGT ATA TCT GGA   450
 F  I  V  K  N  L  S  L  L  I  K  A  A  Q  Q  Q  D  S  G  L  Y  C  L  E  V  T  S  I  S  G                               115

AAA GTT CAG ACA GCC ACG TTC CAG GTT TTT GTA TTT GAT AAA GTT GAG AAA CCC CGC CTA CAG GGG CAG GGG AAG ATC CTG GAC AGA GGG   540
 K  V  Q  T  A  T  F  Q  V  F  V  F  D  K  V  E  K  P  R  L  Q  G  Q  G  K  I  L  D  R  G                               145

AGA TGC CAA GTG GCT CTG TCT TGC TTG GTC TCC AGG GAT GGC AAT GTG TCC TAT GCT TGG TAC AGA GGG AGC AAG CTG ATC CAG ACA GCA   630
 R  C  Q  V  A  L  S  C  L  V  S  R  D  G  N  V  S  Y  A  W  Y  R  G  S  K  L  I  Q  T  A                               175

GGG AAC CTC ACC TAC CTG GAC GAG GAG GTT GAC ATT AAT GGC ACT CAC ACA TAT ACC TGC AAT GTC AGC AAT CCT GTT AGC TGG GAA AGC   720
 G  N  L  T  Y  L  D  E  E  V  D  I  N  G  T  H  T  Y  T  C  N  V  S  N  P  V  S  W  E  S                               205

CAC ACC CTG AAT CTC ACT CAG GAC TGT CAG AAT GCC CAT CAG GAA TTC AGA TTT TGG CCG TTT TTG GTG ATC ATC GTG ATT CTA AGC GCA   810
 H  T  L  N  L  T  Q  D  C  Q  N  A  H  Q  E  F  R  F  W  P  F  L  V  I  I  V  I  L  S  A                               235

CTG TTC CTT GGC ACC CTT GCC TGC TTC TGT GTG TGG AGG AGA AAG AGG AAG GAG AAG CAG TCA GAG ACC AGT CCC AAG GAA TTT TTG ACA   900
 L  F  L  G  T  L  A  C  F  C  V  W  R  R  K  R  K  E  K  Q  S  E  T  S  P  K  E  F  L  T                               265

ATT TAC GAA GAT GTC AAG GAT CTG AAA ACC AGG AGA AAT CAC GAG CAG GAG CAG ACT TTT CCT GGA GGG GGG AGC ACC ATC TAC TCT ATG   990
 I  Y  E  D  V  K  D  L  K  T  R  R  N  H  E  Q  E  Q  T  F  P  G  G  G  S  T  I  Y  S  M                               295

ATC CAG TCC CAG TCT TCT GCT CCC ACG TCA CAA GAA CCT GCA TAT ACA TTA TAT TCA TTA ATT CAG CCT TCC AGG AAG TCT GGA TCC AGG  1080
 I  Q  S  Q  S  S  A  P  T  S  Q  E  P  A  Y  T  L  Y  S  L  I  Q  P  S  R  K  S  G  S  R                               325

AAG AGG AAC CAC AGC CCT TCC TTC AAT AGC ACT ATC TAT GAA GTG ATT GGA AAG AGT CAA CCT AAA GCC CAG AAC CCT GCT CGA TTG AGC  1170
 K  R  N  H  S  P  S  F  N  S  T  I  Y  E  V  I  G  K  S  Q  P  K  A  Q  N  P  A  R  L  S                               355

CGC AAA GAG CTG GAG AAC TTT GAT GTT TAT TCC TAG TTG CTG CAG CAA TTC TCA CCT TTC TTG CAC ATC AGC ATC TGC TTT GGG AAT TGG  1260
 R  K  E  L  E  N  F  D  V  Y  S                                                                                          365

CAC AGT GGA TGA CGG CAC AGG AGT CTC TAT AGA ACA GTT CCT AGT CTG GAG AGG ATA TGG AAA TTT GTT CTT GTT CTA TAT TTT GTT TTG  1350
AAA ATG ATG TCT AAC AAC CAT GAT AAG AGC AAG GCT GTT AAA TAA TAT CTT CCA ATT TAC AGA TCA GAC ATG AAT GGG TGG AGG GGT TAG  1440
GTT GTT CAC AAA AGG CCA CAT TCC                                                                                         1464
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B

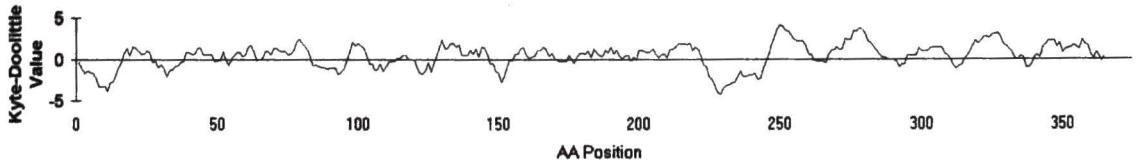
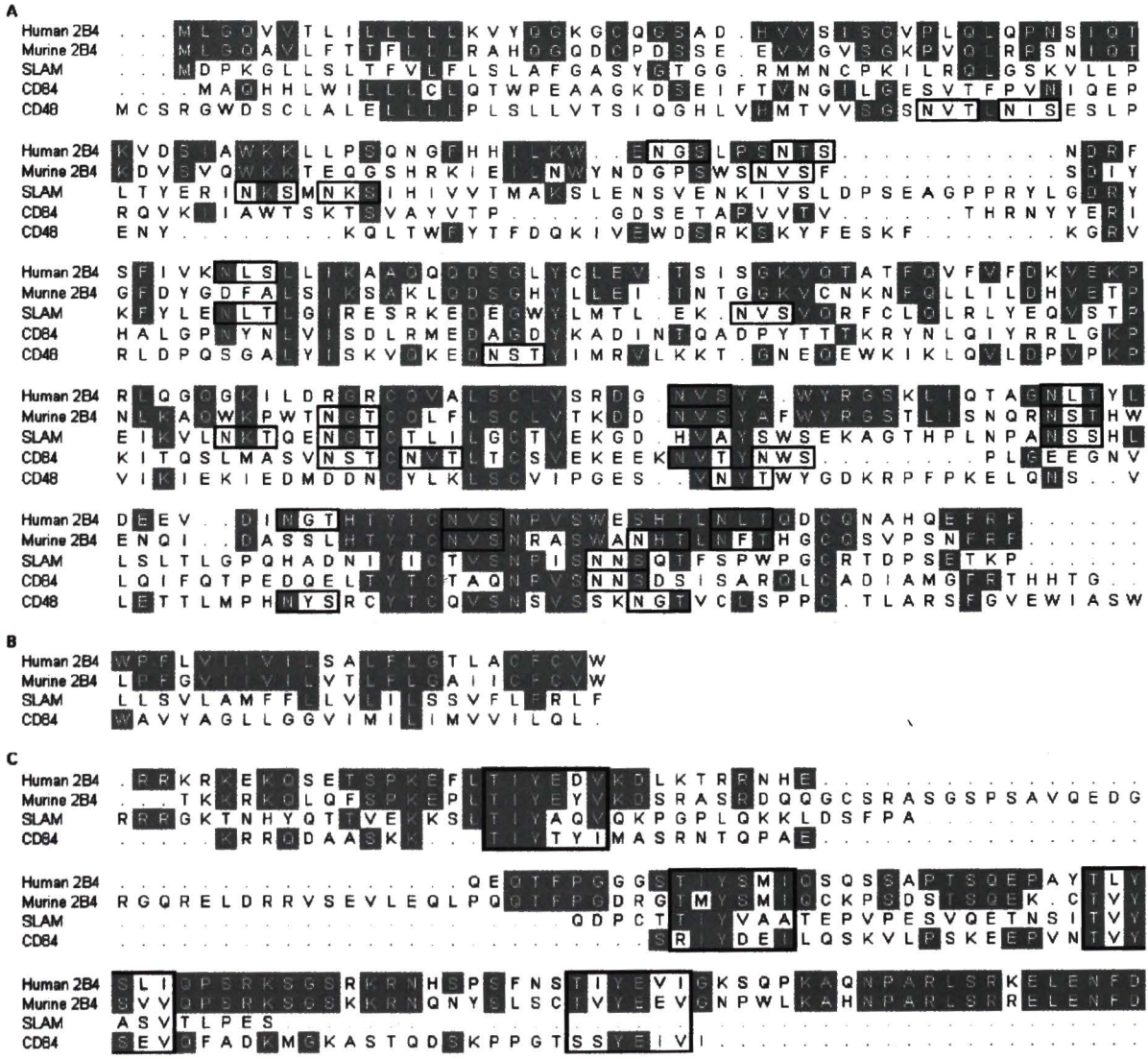


Figure 12. Alignment of amino acid sequences of human 2B4 and other CD2 subfamily receptors. Conserved residues are shaded. *A,* Alignment of the extracellular domains. Glycosylation sites in the extracellular domain are boxed. *B,* Alignment of the transmembrane domains for members that possess them. *C,* Alignment of the intracellular domains. Tyrosine containing motifs in the intracellular domain are boxed. The amino acid comparison was compiled by using the PILEUP program in the Genetics Computer Group software. This figure was reproduced from Kent S. Boles, Marco Colonna, Hideo Nakajima, Samuel S. Chuang, Susan E. Stepp, Michael Bennett, Vinay Kumar, and Porunelloor A. Mathew. 1999. Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4. *Tissue Antigens* 54: 27-34 with permission.


A
Human 2B4
Murine 2B4
SLAM
CD84
CD48
B
Human 2B4
Murine 2B4
SLAM
CD84
C
Human 2B4
Murine 2B4
SLAM
CD84

Expression of human 2B4 in different tissues and cells

The expression of 2B4 transcripts in various cell lines and different human tissues was analyzed by Northern blot of total RNA or poly(A)$^+$ RNA. The full length cDNA hybridizes to transcripts of 3 kb and 5 kb in total RNA from T (Jurkat) and NK (YT) human cell lines (Figure 13A). Additionally, LAK cultures from one healthy donor also expressed transcripts of 3 kb and 5 kb. However, mRNA from human peripheral blood leukocytes, spleen, and lymph node expressed only the 3 kb transcript (Figure 13B). Bone marrow and fetal liver tissues expressed very low levels of transcript that were below the detection level of Northern blot analysis.

Southern analysis of human genomic DNA

Southern blot analysis of human genomic DNA digested with different restriction enzymes (BamH I, EcoR I, Hind III, and Xba I) hybridized to several restriction fragments when probed with a full length cDNA probe (Figure 14). The recent characterization of a mouse 2B4 full length genomic clone showed that the gene is about 27 kb and has nine exons (Stepp et al., 1999b). The sizes of different restriction fragments identified in Figure 14 sum to a gene of about 25 kb in humans. The genomic Southern pattern is similar to the murine gene pattern indicating that there is only a single gene in both mice and humans.

Figure 13. RNA blot analysis of 2B4 transcripts hybridized with a 32P labeled full length 2B4 probe. *A*, Total RNA (20 μg) isolated from the YT and Jurkat cell lines in addition to LAK cell cultures (CD56+ and CD56-) from a healthy donor were electrophoresed in formaldehyde agarose gel, blotted and probed. The position of 28S and 18S rRNA are shown at the left of the panel. *B*, Northern blot of poly(A)+ RNA from spleen, lymph node, thymus, peripheral blood leukocyte, bone marrow, and fetal liver. The sizes of RNA molecular standards are shown at the left of the panel. The panel at the bottom represent s the northern blot stripped and reprobed for β-actin. This figure was reproduced from Kent S. Boles, Marco Colonna, Hideo Nakajima, Samuel S. Chuang, Susan E. Stepp, Michael Bennett, Vinay Kumar, and Porunelloor A. Mathew. 1999. Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4. *Tissue Antigens* 54: 27-34 with permission.

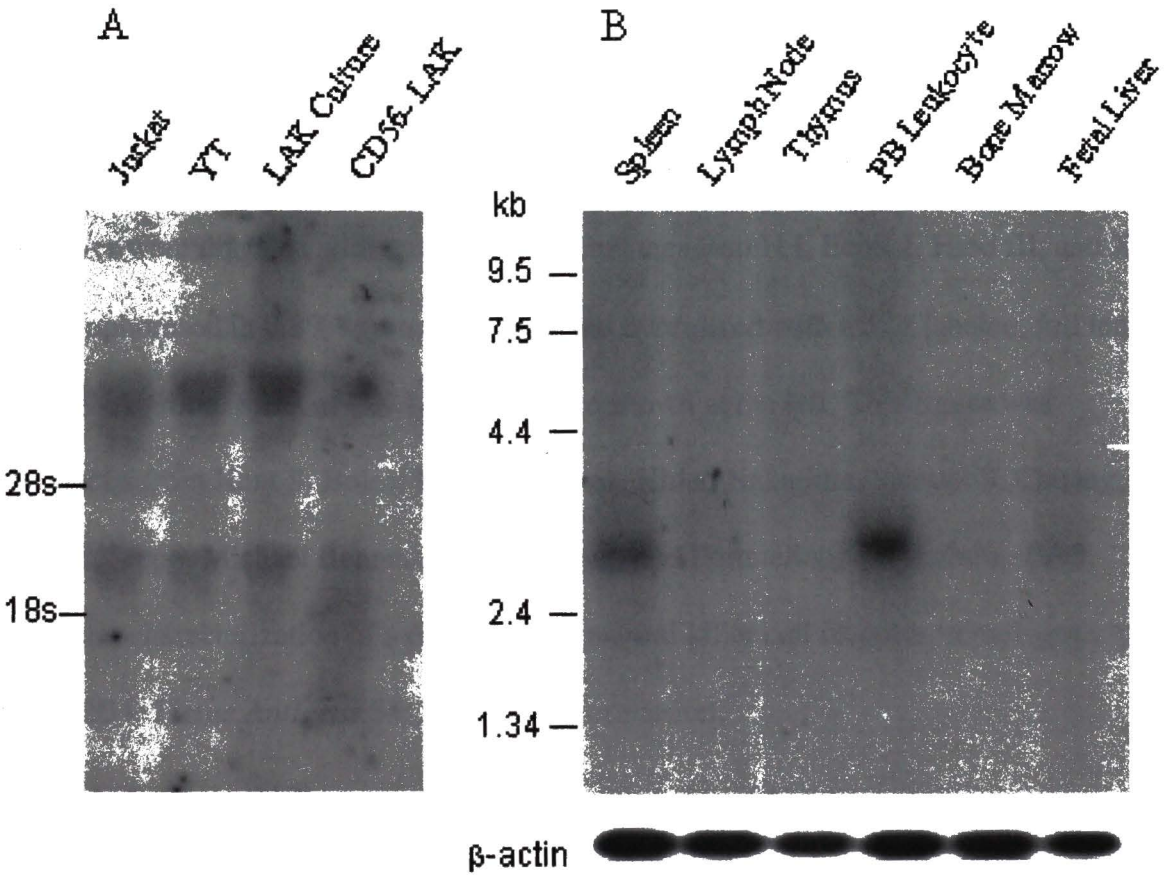
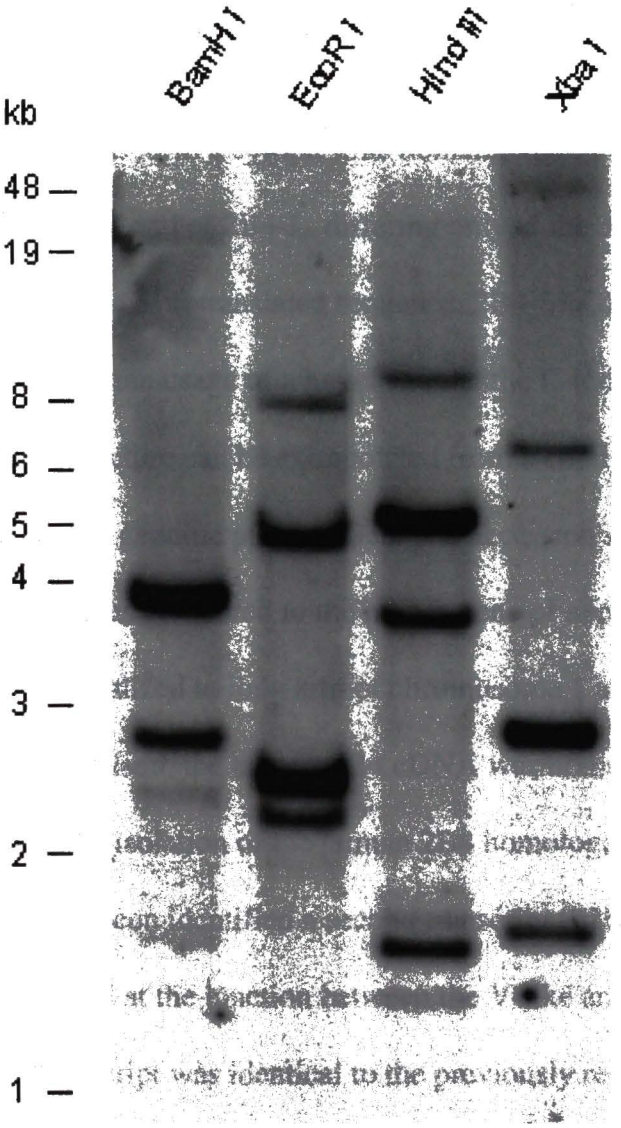

A
Jurkat
YT
LAK Culture
CD56- LAK
28s
18s
B
Spleen
Lymph Node
Thymus
PB Leukocyte
Bone Marrow
Fetal Liver
kb
9.5
7.5
4.4
2.4
1.34
β-actin

Figure 14. Genomic DNA blot analysis of human 2B4. Human genomic DNA (20 μg) from liver were digested with the restriction enzymes BamH I, EcoR I, Hind III, and Xba I, electrophoresed in 0.8% agarose, blotted, and hybridized with a ^{32}P labeled, full length 2B4 cDNA probe. Sizes of DNA standards are shown at the left. This figure was reproduced from Kent S. Boles, Marco Colonna, Hideo Nakajima, Samuel S. Chuang, Susan E. Stepp, Michael Bennett, Vinay Kumar, and Porunelloor A. Mathew. 1999. Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4. *Tissue Antigens* 54: 27-34 with permission.

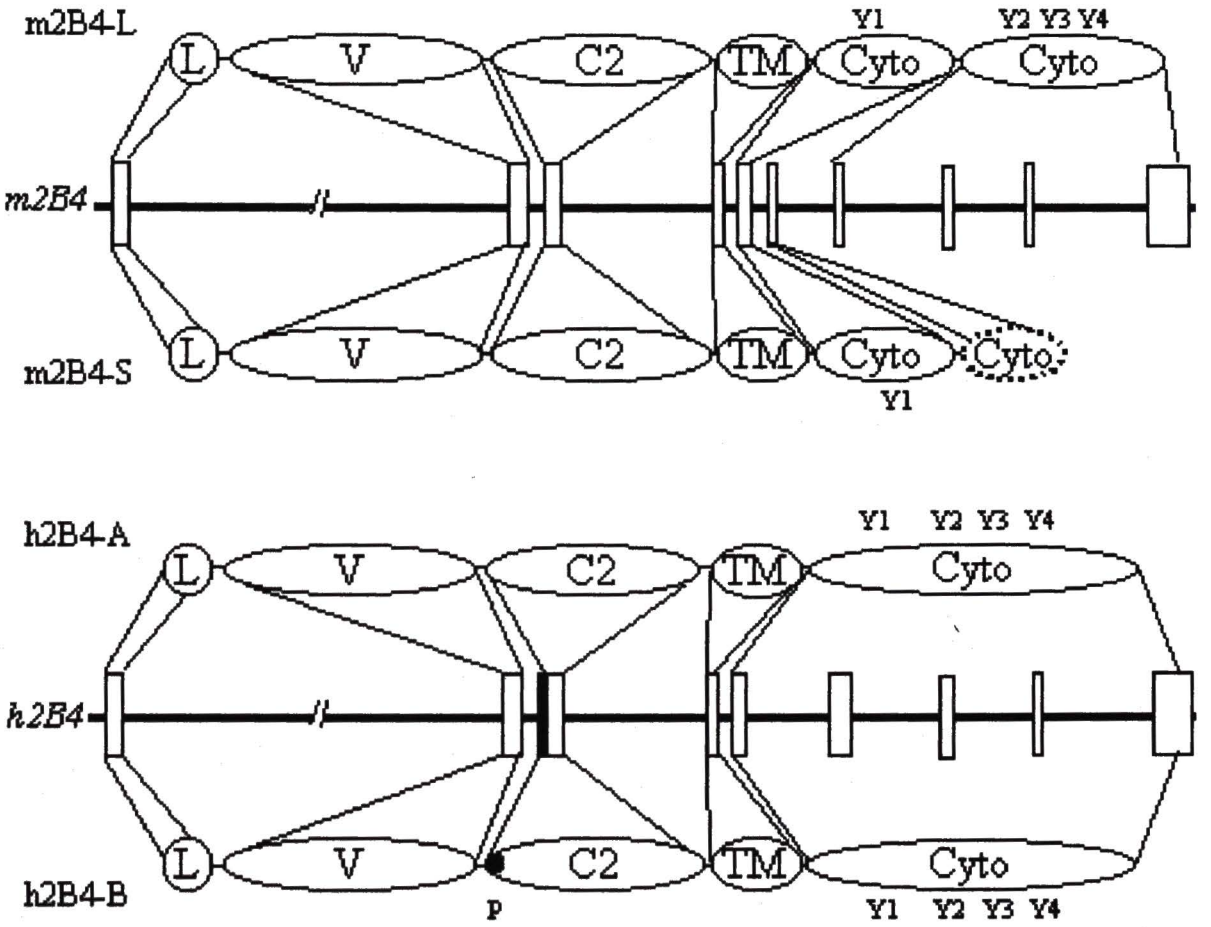

BamH I
EcoR I
Hind III
Xba I
kb
48
19
8
6
5
4
3
2
1

2B4 gene characterization

The murine *2B4* gene consists of at least 9 exons with one exon dedicated to the leader sequence, V-like, C2-like, and transmembrane domains, each (Figure 15). The first intron is rather large at approximately 16 kb giving rise to a total gene size of about 27 kb. Variable exon usage gives rise to the two forms of 2B4 in the mouse (Stepp et al., 1999b). 4 exons encode the 2B4-L form's cytoplasmic domain, giving rise to 4 tyrosine motifs. 2B4-S is identical to the 5' end of 2B4-L, differing only at the 3' end in a portion of the cytoplasmic domain and the 3' untranslated sequence. 2B4-S is the product of the same first 5 exons in 2B4-L with the usage of a novel exon at the C-terminal.

The *human 2B4* gene structure can be extrapolated from a contiguous gene sequence produced by the human genome project (Genbank accession number AL121958). It is 29 kb in length as compared to the mouse gene of approximately 27 kb. Additionally, the gene can be localized to long arm of chromosome 1 at approximately 1q23. Based on sequence comparison, the human 2B4 cDNA was homologous to the m2B4-L form. Subsequent to the isolation of the human 2B4 homologue, an additional isoform of the 2B4 receptor has been identified a second transcript (h2B4-B) with an additional 15 nucleotides inserted at the junction between the V-like and C2-like domains (Figure 15). Otherwise, the transcript was identical to the previously reported clone (Kumaresan and Mathew, 2000).

Figure 15. Schematic representation of the genomic organization of the exons and introns for murine *2B4* and human *2B4* genes. Exons are represented by rectangles. Protein domains of the splice variants are indicated by L, V, C2, TM, and Cyto and refer to leader sequence, Ig V-like domain, Ig C2-like domain, transmembrane domain, and cytoplasmic domain, respectively. Y1-Y4 represents tyrosine containing motifs and the proline added to the human 2B4-B form is shown by a P.

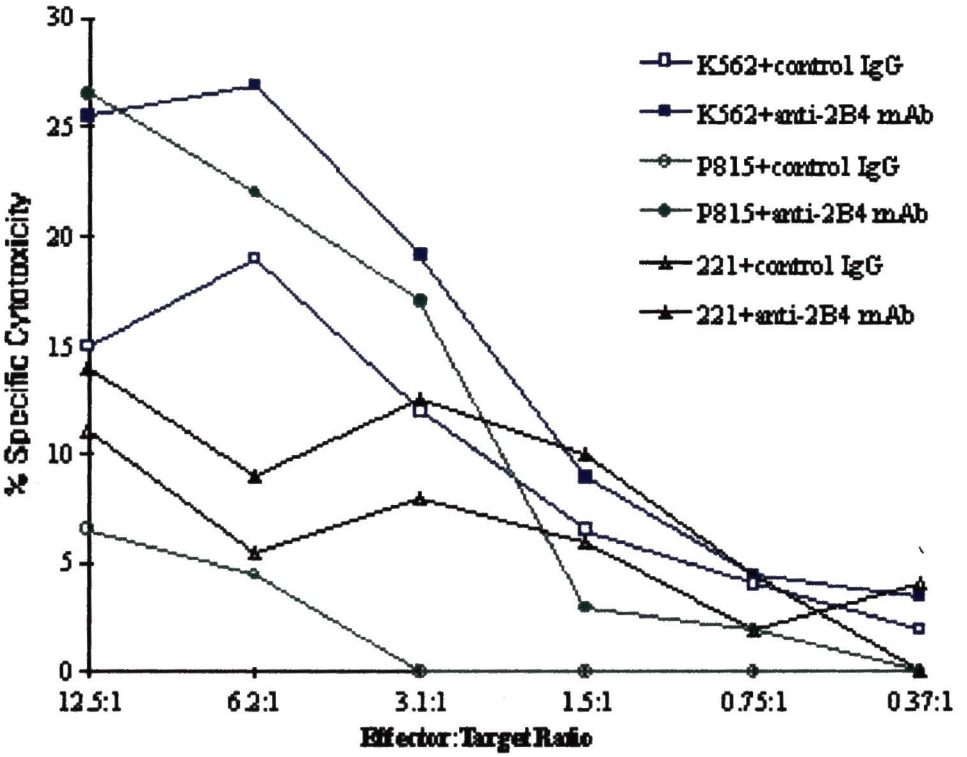

m2B4-L
L
V
C2
TM
Y1
Cyto
Y2 Y3 Y4
Cyto
m2B4
m2B4-S
L
V
C2
TM
Cyto
Cyto
Y1
h2B4-A
Y1 Y2 Y3 Y4
L
V
C2
TM
Cyto
h2B4
h2B4-B
L
V
C2
TM
Cyto
P
Y1 Y2 Y3 Y4

Characterization of the human *2B4* gene revealed a similar structure to the mouse gene (Figure 15). The human 2B4 gene contains nine exons, the first four exons encode the leader sequence, V-like, C2-like, and transmembrane domains and the remaining five exons encode the cytoplasmic domain. Furthermore, the first intron is also very large at approximately 17 kb. The difference between h2B4-A and h2B4-B arises from differential splicing of exon 3. Exon 3 contains an internal splice site and the first 15 nucleotides of exon 3 are spliced out of h2B4-A. h2B4-B contains the 15 nucleotides which results in the additional 5 amino acids, including a proline residue. The difference in the extracellular domains of these two receptors might mediate differential ligand interaction or affinity. Because the intracellular domains are identical, they most likely deliver similar signals.

Assay for lytic activity

In order to determine the functional role of 2B4 in the killing of targets by NK cells, Samuel Chang and I determined the lytic activity of activated human NK cells against various target cells using a standard chromium release assay in the presence of a mAb that recognizes human 2B4 (Figure 16). The lytic activity of human polyclonal NK cells was augmented in the presence of anti-2B4 mAb against P815, K562, as well as 721.221 target cells compared with a control human IgG. Because 721.221 is MHC class I deficient, activation of human NK cytolytic activity via 2B4 is independent of the expression of class I molecules on target cells. This suggests that 2B4 can transduce activation signals to human NK cells.

Figure 16. Cytolytic activity of human polyclonal NK cells against various targets in presence of anti-2B4 mAb. Cytotoxicity was determined in a standard 4 hr ^{51}Cr release assay. The figure depicts a representation of several independent trials. This figure was reproduced from Kent S. Boles, Marco Colonna, Hideo Nakajima, Samuel S. Chuang, Susan E. Stepp, Michael Bennett, Vinay Kumar, and Porunelloor A. Mathew. 1999. Molecular characterization of a novel human natural killer cell receptor homologous to mouse 2B4. *Tissue Antigens* 54: 27-34 with permission.


30
25
20
15
10
5
0
% Specific Cytotoxicity
12.5:1
6.2:1
3.1:1
1.5:1
0.75:1
0.37:1
Effector:Target Ratio
K562+control IgG
K562+anti-2B4 mAb
P815+control IgG
P815+anti-2B4 mAb
221+control IgG
221+anti-2B4 mAb

CHAPTER 4

IDENTIFICATION OF AN ADDITIONAL MEMBER OF THE CD2 SUBSET CONTAINING THE NOVEL 2B4 TYROSINE MOTIF

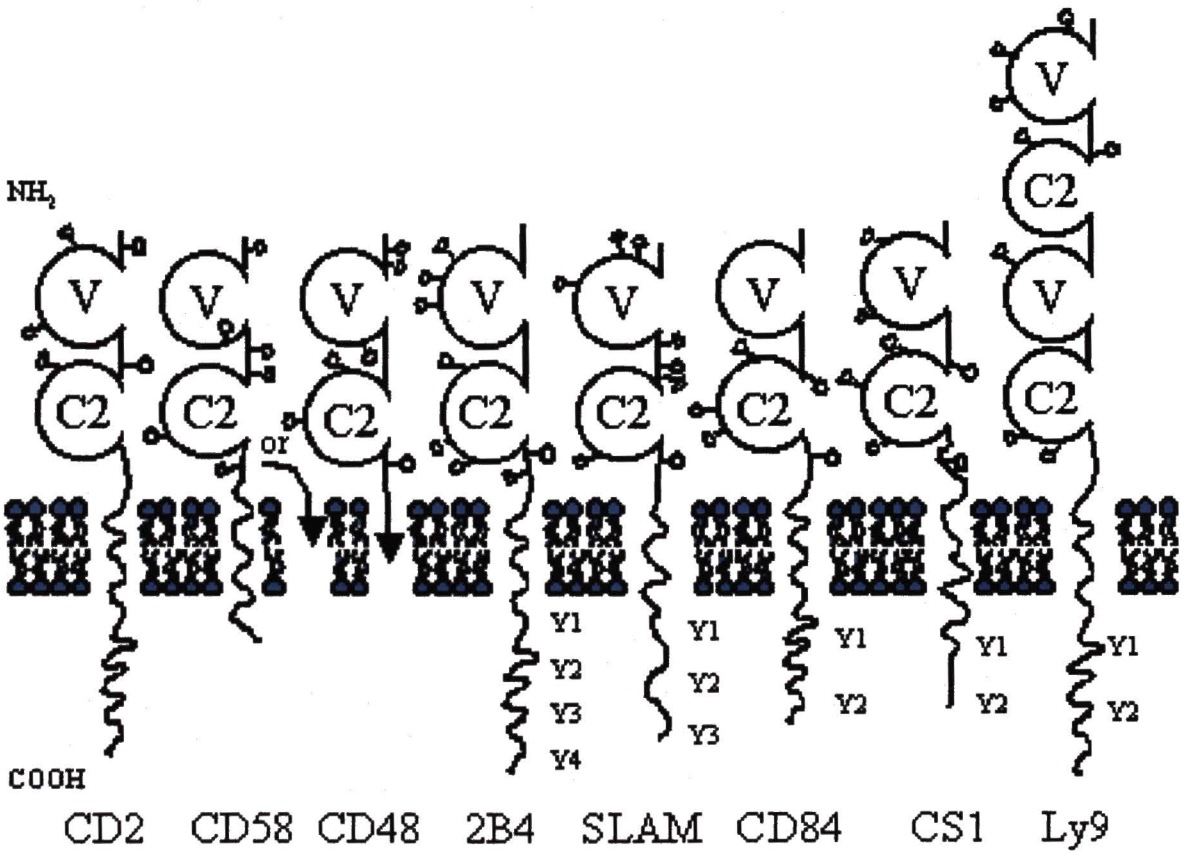
Introduction

Natural killer (NK) cells constitute the third major population of lymphocytes. They are capable of lysing target cells in addition to regulating both innate and adaptive immune responses (Trincheri, 1989; (Lanier, 2000). The activation of NK cells is the sum of receptor signaling from both inhibitory and stimulatory receptors (Lanier, 2000); Tomasello et al 2000a). NK receptors are often arranged in gene clusters due to gene duplication. These families include members that are either activating or inhibitory. These signaling differences arise from variance within the transmembrane and cytoplasmic domains. A common denominator of inhibitory receptors within these families is the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tails with the consensus sequence of I/V/L/SxYxxL/V (Long, 1999). The inhibitory signaling is mediated by binding of the protein tyrosine phosphatases SHP-1, SHP-2, and SHIP to the phosphorylated tyrosines. Activating receptors within these families lack the ITIM motif and have charged residues within their transmembrane domains that allow association with adaptor molecules such as FcεRIγ (Tomasello et al., 2000a; Weiss and Littman, 1994) or TYROBP (formerly known as DAP12; Paloneva et

al., 2000). These adapters contain an immunoreceptor tyrosine-based activation motif (ITAM) and consequently transduces stimulatory signals via association with Src family kinases (Gaul et al., 2000; Visco et al., 2000). The consensus sequence for the ITAM is I/VxYxxL/Vx_{26-31}I/VxYxxL/V (Tomasello et al., 2000a; Weiss and Littman, 1994).

Members of the CD2 subset of the immunoglobulin (Ig) superfamily of cell surface receptors, most notably, 2B4 and SLAM (CDw150), are expressed on lymphocytes and involved in cellular activation such as lymphoproliferation, cytokine production, cytotoxicity, and invasiveness (Aversa et al., 1997a; Aversa et al., 1997b; Boles et al., 1999b; Chuang et al., 2000; Mathew et al., 1993; Punnonen et al., 1997; Wang et al., 2000). These receptors have novel tyrosine containing motifs in their cytoplasmic domains (TxYxxI/V/A) (Figure 17) (Boles et al., 1999b; Cocks et al., 1995). CD84, another member of the CD2 subset, also contains the consensus tyrosine motif, but its function remains to be determined (de la Fuente et al., 1997). Unlike many NK cell receptors that bind MHC class I molecules as their ligands (Tomasello et al., 2000a), CD2 subfamily members bind homophilically to members of the CD2 subfamily (Davis et al., 1998). CD2 binds CD58 and CD48 in the human and CD48 in the rodent (Arulanandam et al., 1993; Kato et al., 1992; Sandrin et al., 1993; van der Merwe et al., 1994; van der Merwe et al., 1993). Additionally, SLAM is a self ligand leading to activation of lymphocytes (T and B cells) (Punnonen et al., 1997). CD48 is a high affinity ligand for 2B4 (Brown et al., 1998; Latchman, McKay and Reiser, 1998).CD48 is widely expressed in leukocytes and its soluble form is detectable in the blood (Smith et al., 1997).

Figure 17. Members of the CD2 subset of receptors. The subset members have an N-terminal domain which is extracellular. Some of the receptors are GPI-linked to the surface membrane, which is indicated by an arrow. Putative N-linked glycosylation sites in the extracellular domains are indicated . Y1-Y4 represents tyrosine containing motifs shown in the intracellular domains.


NH2
V
C2
or
Y1
Y2
Y3
Y4
COOH
CD2
CD58
CD48
2B4
SLAM
CD84
CS1
Ly9

The novel tyrosine motifs in SLAM and 2B4 have both been shown to interact with the src homology 2 (SH2) domain of the SLAM associated protein (SAP) and it may regulate their signaling (Sayos et al., 1998; Tangye et al., 1999). Mutations in the SH2 domain of SAP have been identified as the genetic basis for X-linked lymphoproliferative disease (XLP) (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998). Recently, Parolini et al. reported that the 2B4 receptor transduces inhibitory signals in XLP patients when stimulated by CD48 positive B cells (Parolini et al., 2000). Taken together, these data suggest that the cause of XLP is complex and probably due to dysregulation of phosphorylation dependent interactions at multiple sites, including receptors and cytoplasmic adaptor molecules. In addition to signaling via 2B4 and SLAM, other receptors that contain the unique tyrosine motif might also contribute to the immune dysregulation seen in XLP.

The genes that encode the CD2 family of receptors are located on human chromosome 1 in two complexes. *2B4*, *SLAM*, *CD48*, *Ly9* and *CD84* are located on the long arm of the chromosome at 1q21-24 (Aversa et al., 1997a; Boles and Mathew, 2001; de la Fuente et al., 1997; Kingsmore et al., 1995; Sandrin et al., 1996; Staunton et al., 1989; Tangye et al., 1999). The murine genes for *2B4*, *CD48*, *Ly9*, *Ly108* and *CD84* are located on the syntenic region of the long arm of chromosome 1, as well (de la Fuente et al., 1999; Kingsmore et al., 1995; Kubota et al., 1999). The genes for *CD2* and *CD58* are located on the short arm of human chromosome 1 at 1p13 (Clayton et al., 1988; Davis et al., 1998; Sewell et al., 1988) and the murine *CD2* gene is located on chromosome 3 (Clayton et al., 1988).

Methods

EST database search and cDNA library screening

The EST (expressed sequence tag) database at Genbank (http://www.ncbi.nlm.nih.gov) was searched with the TblastN program versus a consensus sequence of human the CD2 subset of receptors. Several overlapping clones were identified and oligonucleotide primers (CS1 F1, 5'-cctcccatggtcctcctgtg-3', CS1 R1, 5'–gagacttaggggagtgcactgctg-3') were designed to amplify a 363 bp fragment within the cytoplasmic tail. cDNA from a NK cell library constructed in λ phage by Dr. J. Houchins (R & D Systems, Minneapolis, MN and kindly provided by Dr. A. Brooks, NIH, Bethesda, MD) was successfully used as template. PCR cycle conditions were 94° C for 30 seconds, 50° C annealing temperature for 30 seconds, and a 72° C extension for 45 seconds repeated for 30 cycles using *Taq* DNA polymerase from GIBCO BRL (Grand Island, NY) at 2 mM $MgCl_2$. The same library was then screened with the resulting PCR fragment labeled with α-^{32}P dCTP (Feinberg and Vogelstein, 1983; Sambrook, Fritch and Maniatis, 1989). Approximately, $5x10^5$ clones were screened. After three rounds of screening, phage DNA was isolated from positive clones by the method of Lee et al. (Lee and Clark, 1997a). All positively selected clones were sequenced (Automated sequencing facility, Department of Pathology, UT Southwestern Medical Center, Dallas) and analyzed (Genetics Computer Group, Wisconsin package). One clone (C9C1A), which contained an open reading frame, was identified for further study. The transcript was

named CS1 (CD2 subset 1) due to its similarity to other members of the CD2 subset of receptors.

Cell culture

Human tumor cell lines Jurkat (T cell), YT (NK cell), HL-60 (monocytic), and DB (B cell), in addition to a murine lymphoma cell line (YAC-1), were cultured in RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/ml of penicillin and streptomycin, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Gibco BRL, Grand Island, NY). All cell lines were grown to one million per ml and split 1:2 twenty-four hours before RNA isolation. PBMC were isolated from 60 ml of venous blood from a healthy donor by Ficol-Paque centrifugation (Pharmacia, Piscataway, NJ).

RNA and DNA blot analysis

Total RNA was isolated with the RNAstat 60 reagent according to the manufacturer's protocol (Teltest Inc., Friendswood, TX), divided into 20 μg aliquots, and stored in 70% EtOH at -80° C until used. 1% agarose gels for Northern analysis were stained with Ethidium bromide after electrophoresis to insure equal loading by comparison of rRNA. Northern blots were probed with 25 ng of the full length cDNA labeled with α^{32}P dCTP (Feinberg and Vogelstein, 1983; Sambrook, Fritch and Maniatis, 1989). The first blot consisted of 20 μg of total RNA from human monocytic, T, B, and NK cell lines (HL-60, Jurkat, DB, and YT, respectively), a mouse cell line (YAC-1), and

PBMC cells from a healthy donor immobilized on Hybond nylon (Amersham, Arlington Heights, IL). Prehybridization and hybridizations were performed according to instructions of Amersham for the Hybond nylon membrane at 65° C. The second membrane was purchased from Clontech (Palo Alto, CA) and contained mRNA samples from human spleen, lymph node, thymus, peripheral blood leukocytes, bone marrow and fetal liver (Human Immune System Multiple Tissue Northern Blot II). It was hybridized per the manufacturer's directions with the included ExpressHyb Hybridization solution at 65° C. Blots were exposed to Hyperfilm (Amersham, Arlington Heights, IL). The membranes were subsequently stripped and reprobed for β actin in insure equal loading.

Genomic DNA was isolated from human liver as per standard protocol (Sambrook, Fritch and Maniatis, 1989). For DNA blot analysis, human genomic DNA samples (20 μg each) were digested with various restriction enzymes (BamH I, EcoR I, Hind III, and Xba I) and separated on 0.8% agarose gel by electrophoresis. The DNA was transferred to Hybond nylon membrane under alkaline condition (0.4N NaOH) and fixed by UV cross linking. The membrane was prehybridized for 2 hours at 65°C in hybridization buffer (1 mM EDTA, 0.5 M sodium phosphate, pH 7.2, 7% SDS, 100 mg/ml ssDNA). The probe (50 ng of the full length cDNA labeled with α^{32}P dCTP) was added to the same buffer and hybridizations continued for 18 hr at 65° C (Feinberg and Vogelstein, 1983; Sambrook, Fritch and Maniatis, 1989). The membrane was washed with a buffer containing 40 mM sodium phosphate, pH 7.2, 1% SDS at 65° C for 1 hr. The filter was exposed to Hyperfilm for one day (Amersham, Arlington Heights, IL) and developed.

CS1-FLAG construct production

A FLAG epitope was introduced into the N-terminal of the CS1 receptor to allow antibody cross-linking of the receptor on the cell surface. PCR primers were used to amplify the cDNA without the leader sequence and the product was cloned into the pFLAG-CMV3 expression vector with contains a leader sequence (trypsin) and a FLAG epitope under the expression of a CMV promoter (Figure 24A). The primers were CS1 EcoR F1 5'- ATT TGA ATT CTT TCT GAA GAG AGA GAG AC-3' and CS1 Xba R1 5'-TTG ACT CGA GAC TTA GGG GAG TGC ACT GC-3'. The CS1 cDNA fragment was cloned in frame into the expression vector with EcoR I and Xba I sites that were introduced via the PCR primers. The constructs were prepared by CsCl centrifugation and linearized with the Pvu I restriction endonuclease. Subsequently, they were transfected into either BW or YT cells with the DMRIE-C lipid reagent (Gibco Life Technologies). Clones were selected for three weeks under the selection of geneticin (Sigma) at 500 μg / ml. Flow cytometry was used to confirm surface expression (Figure 24C) and clones were selected for further analysis.

Assay for lytic activity

The human NK cell line YT alone or expressing the CS1-FLAG construct was tested for the ability to lyse K562 and P815 target cells in a 4 hour ^{51}Cr release assay as described previously (Tutt et al., 1986). The effector cells were incubated for 30 minutes with a control or specific antibodies in 96 well, round bottom plates prior to the addition

of ^{51}Cr labeled target cells. A FLAG monoclonal antibody added as a specific Ab. Plates were subsequently incubated at 37° C for four hours. The plates were spun at 250 x g for 7 minutes to pellet the cells and 100 µl of the supernatant was removed and counted by scintillation counting. Percent cytotoxicity was calculated as described (Tutt et al., 1986).

Interferon γ release assay

YT or YT expressing the CS1-FLAG construct were incubated in the presence of K562. One half million YT and K562 each were combined into a volume of 1 ml. Control antibody (HA), FLAG, or C1.7 antibodies were added at 200 ng/ml and incubated for 18 hours at 37° C. 100 ml of the supernatant of each well was removed and tested for IFN-γ by ELISA (Amersham, UK). All conditions were repeated in triplicate.

Results

Isolation and sequence analysis of the CS1 cDNA clone

The regulation of the immune response in addition to the pathogenesis of diseases such as XLP might include additional receptors of the CD2 subset containing the unique tyrosine motif TxYxxI/V/A. In order to identify any novel molecules that contain the unique tyrosine motif, I searched the EST database with a sequences of 2B4, SLAM, and CD84 cytoplasmic domains. Several overlapping clones were identified. I designed oligonucleotide primers based on the identified sequences and PCR amplified a 363 bp fragment that encompasses the novel tyrosine motifs. cDNA isolated from a human NK cell library (constructed by Dr. J. Houchins, R & D Systems, Minneapolis, MN and

kindly provided by Dr. A. Brooks, NIH, Bethesda, MD) was used as the template. In order to isolate a full length clone , I screened the same library with the 363 bp PCR product labeled with α-^{32}P dCTP. A positive clone (C9C1A) was selected for further analysis. It contained a cDNA insert of 1083 bp with an open reading frame encoding a polypeptide of 335 amino acid residues (Genbank accession number AF291815, Figure 18A). The predicted protein sequence had a single transmembrane domain of 25 amino acid residues (Figure 18B) and an intracellular domain of 85 amino acid residues. Additionally, it had an extracellular domain of 225 amino acid residues which contained seven putative N-linked glycosylation sites The homology of the predicted protein sequence of CS1 indicates that it is a member of the Ig superfamily. Furthermore it is a new member of the CD2 subset of receptors. It has the highest similarities to CD84, SLAM, and 2B4 with 47, 44, and 40% similarity, respectively (Figure 19). Alignment of the CS1 putative protein indicates a similar structure with many conserved residues compared to other CD2 subset receptors. The cytoplasmic region contains two of the novel tyrosine motifs similar to those in 2B4 and SLAM. These are indicated by a threonine residue in the –2 position relative to the tyrosine (TxYxxI/V). There is an additional tyrosine containing motif near the C-terminal of the CS1 receptor. The –2 position of that motif is phenylalanine (FxYxxV) and thus do not belong to any known consensus sequence motifs.

Figure 18. Nucleotide sequence of CS1 cDNA and the predicted amino acid sequence. ***A***, The nucleotide sequence and the putative peptide of CS1 (Genbank accession AF291815). The signal peptide is underlined. The transmembrane domain is double underlined. Glycosylation sites in the extracellular domain are boxed. Tyrosine containing motifs in the intracellular domain are shaded. ***B***, Hydrophilicity plot of the CS1 putative peptide sequence determined by the Kyte-Doolittle method. This figure was reproduced from Kent S. Boles and Porunelloor A. Mathew. 2001. Molecular cloning of CS1, a novel human NK cell receptor belonging to the CD2 subset of the immunoglobulin superfamily. *Immunogenetics* 52: 302-307 with permission.

A

```
GAG AGC AAT ATG GCT GGT TCC CCA ACA TGC CTC ACC CTC ATC TAT ATC CTT TGG CAG CTC ACA GGG TCA GCA GCC TCT GGA CCC GTG AAA   90
          M   A   G   S   P   T   C   L   T   L   I   Y   I   L   W   Q   L   T   G   S   A   A   S   G   P   V   K    26

GAG CTG GTC GGT TCC GTT GGT GGG GCC GTG ACT TTC CCC CTG AAG TCC AAA GTA AAG CAA GTT GAC TCT ATT GTC TGG ACC TTC AAC ACA  180
  E   L   V   G   S   V   G   G   A   V   T   F   P   L   K   S   K   V   K   Q   V   D   S   I   V   W   T   F  [N  T]  56

ACC CCT CTT GTC ACC ATA CAG CCA GAA GGG GGC ACT ATC ATA GTG ACC CAA AAT CGT AAT AGG GAG AGA GTA GAC TTC CCA GAT GGA GGC  270
[T] P   L   V   T   I   Q   P   E   G   G   T   I   I   V   T   Q   N   R   N   R   E   R   V   D   F   P   D   G   G    86

TAC TCC CTG AAG CTC AGC AAA CTG AAG AAG AAT GAC TCA GGG ATC TAC TAT GTG GGG ATA TAC AGC TCA TCA CTC CAG CAG CCC TCC ACC  360
  Y   S   L   K   L   S   K   L   K   K  [N  D  S]  G   I   Y   Y   V   G   I   Y   S   S   S   L   Q   Q   P   S   T  116

CAG GAG TAC GTG CTG CAT GTC TAC GAG CAC CTG TCA AAG CCT AAA GTC ACC ATG GGT CTG CAG AGC AAT AAG AAT GGC ACC TGT GTG ACC  459
  Q   E   Y   V   L   H   V   Y   E   H   L   S   K   P   K   V   T   M   G   L   Q   S   N   K  [N  G  T]  C   V   T  146

AAT CTG ACA TGC TGC ATG GAA CAT GGG GAA GAG GAT GTG ATT TAT ACC TGG AAG GCC CTG GGG CAA GCA GCC AAT GAG TCC CAT AAT GGG  540
[N  L  T]  C   C   M   E   H   G   E   E   D   V   I   Y   T   W   K   A   L   G   Q   A   A  [N  E  S]  H  [N  G]  176

TCC ATC CTC CCC ATC TCC TGG AGA TGG GGA GAA AGT GAT ATG ACC TTC ATC TGC GTT GCC AGG AAC CCT GTC AGC AGA AAC TTC TCA AGC  630
[S]  I   L   P   I   S   W   R   W   G   E   S   D   M   T   F   I   C   V   A   R   N   P   V   S   R  [N  F  S]  S   206

CCC ATC CTT GCC AGG AAG CTC TGT GAA GGT GCT GCT GAT GAC CCA GAT TCC TCC ATG GTC CTC CTG TGT CTC CTG TTG GTG CCC CTC CTG  720
  P   I   L   A   R   K   L   C   E   G   A   A   D   D   P   D   S   S   M   V   L   L   C   L   L   L   V   P   L   L  236

CTC AGT CTC TTT GTA CTG GGG CTA TTT CTT TGG TTT CTG AAG AGA GAG AGA CAA GAA GAG TAC ATT GAA GAG AAG AAG AGA GTG GAC ATT  810
  L   S   L   F   V   L   G   L   F   L   W   F   L   K   R   E   R   Q   E   E   Y   I   E   E   K   K   R   V   D   I  266

TGT CGG GAA ACT CCT AAC ATA TGC CCC CAT TCT GGA GAG AAC ACA GAG TAC GAC ACA ATC CCT CAC ACT AAT AGA ACA ATC CTA AAG GAA  900
  C   R   E   T   P   N   I   C   P   H   S   G   E   N   T   E   Y   D   T   I   P   H   T   N   R   T   I   L   K   E  296

GAT CCA GCA AAT ACG GTT TAC TCC ACT GTG GAA ATA CCG AAA AAG ATG GAA AAT CCC CAC TCA CTG CTC ACG ATG CCA GAC ACA CCA AGG  990
  D   P   A   N   T   V   Y   S   T   V   E   I   P   K   K   M   E   N   P   H   S   L   L   T   M   P   D   T   P   R  326

CTA TTT GCC TAT GAG AAT GTT ATC TAG ACA GCA GTG CAC TGC CCC TAA GTC TCT GCT CAA AAA AAA AAC AAT TCT CGG CCC AAA GAA AAC 1080
  L   F   A   Y   E   N   V   I                                                                                        335
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B

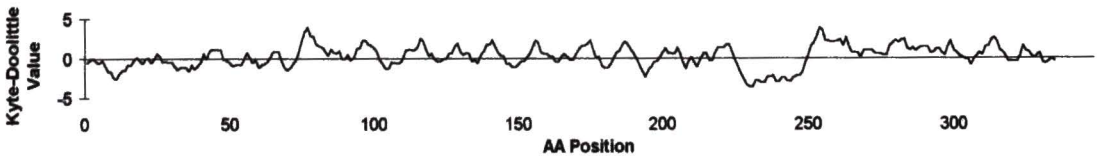
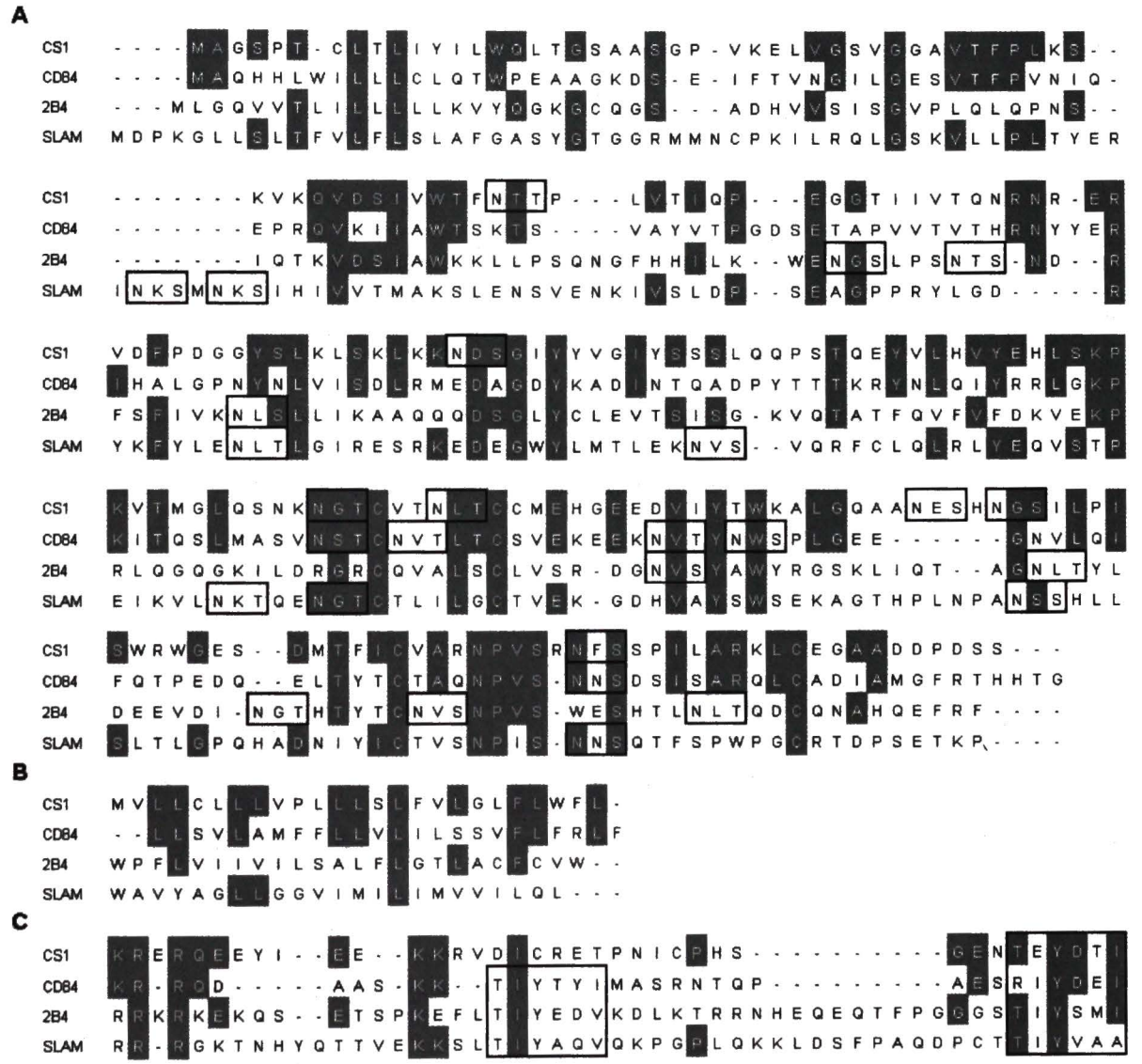


Figure 19. Alignment of amino acid sequences of CS1 and related receptors in the CD2 subset. Residues identical to CS1 are shaded and glycosylation sites are boxed. ***A***, Alignment of the extracellular domains. ***B***, Alignment of the transmembrane domains. ***C***, Alignment of the intracellular domains. The amino acid comparison was compiled by using the PILEUP program in the Genetics Computer Group software. This figure was reproduced from Kent S. Boles and Porunelloor A. Mathew. 2001. Molecular cloning of CS1, a novel human NK cell receptor belonging to the CD2 subset of the immunoglobulin superfamily. *Immunogenetics* 52: 302-307 with permission.


A
CS1
CD84
2B4
SLAM
B
CS1
CD84
2B4
SLAM
C
CS1
CD84
2B4
SLAM

Expression of CS1 in different tissues and cells

The expression of CS1 transcripts in various cell lines and different human tissues was analyzed by northern blotting of total RNA or poly(A)$^+$ RNA. The full length cDNA hybridized to a transcript of approximately 3 kb in total RNA from both a human NK cell line (YT) and PBMC of a healthy donor (Figure 20A). Tissue distribution of CS1 showed that human peripheral blood leukocytes, lymph node, and spleen expressed a transcript of the same relative sizes (Figure 20B). Additionally, a weak signal was detected in the bone marrow sample. The relative expression of the CS1 transcript in lymph node and spleen suggests that it may be expressed in other lymphocyte populations. The relative distribution and regulation of the transcript remains to be determined.

Southern analysis of genomic DNA and chromosomal localization of CS1

The genomic organization of both the mouse and human *2B4* genes have been characterized (Kumaresan and Mathew, 2000; Stepp et al., 1999b) These genes are relatively large (~25 kb) and contain an exceptionally large first intron of 17 kb. Southern analysis indicated that *CS1* gene is approximately 13 kb, which is much smaller than *2B4* (Figure 21). Briefly, genomic DNA was isolated from human liver and digested with four different restriction enzymes (*BamH* I, *EcoR* I, *Hind* III, and *Xba* I), separated on an agarose gel and transferred to a nylon membrane. The full length CS1 cDNA hybridized to several restriction fragments.

Figure 20. RNA blot analysis of the CS1 transcript hybridized with 32P labeled, full length CS1 cDNA. ***A***, A membrane containing total RNA (20 μg) isolated from the YAC-1, HL-60, DB, Jurkat, and YT tumor cell lines. Additionally, a sample was included from the PBMC of a healthy donor. ***B***, Northern blot of poly (A)+ RNA from spleen, lymph node, thymus, peripheral blood leukocytes, bone marrow, and fetal liver tissues. The membrane was a Human Immune System Multiple Tissue Northern Blot II purchased from Clontech (Palo Alto, CA). Both membranes were stripped and hybridized with a β-actin probe. The position of the RNA molecular standards are shown in the center. This figure was reproduced from Kent S. Boles and Porunelloor A. Mathew. 2001. Molecular cloning of CS1, a novel human NK cell receptor belonging to the CD2 subset of the immunoglobulin superfamily. *Immunogenetics* 52: 302-307 with permission.

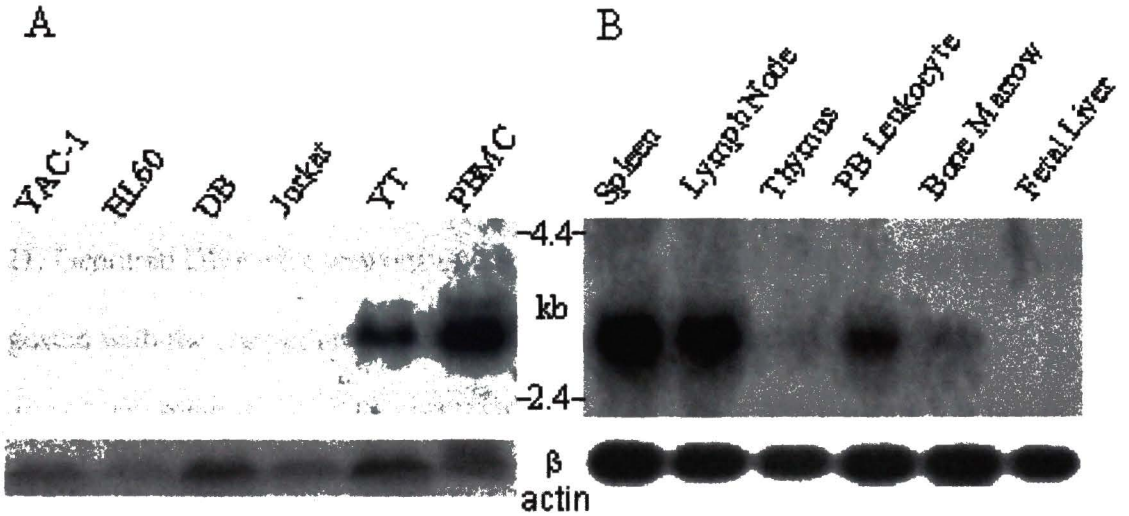
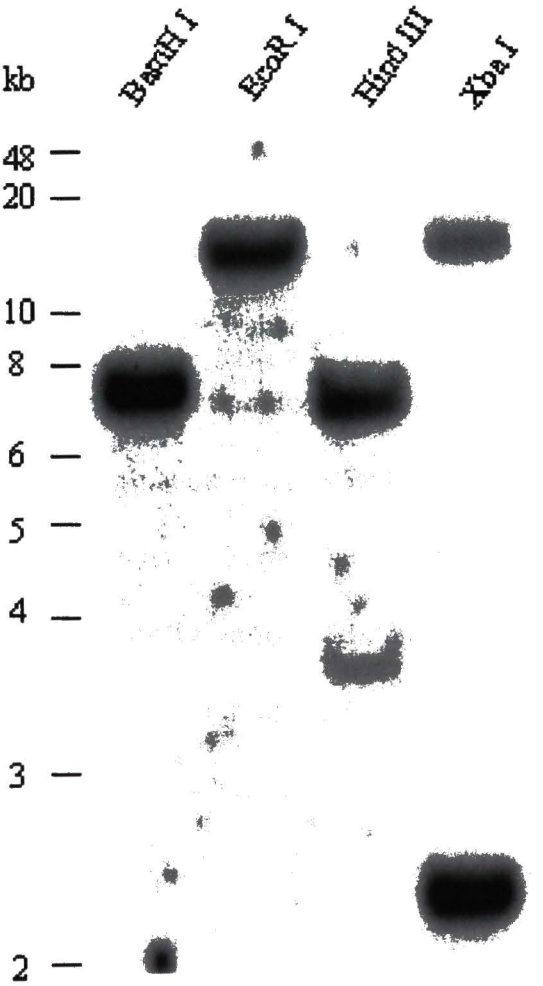

A
B
YAC-1
HL60
DB
Jurkat
YT
PBMC
Spleen
Lymph Node
Thymus
PB Leukocyte
Bone Marrow
Fetal Liver
-4.4-
kb
-2.4-
β
actin

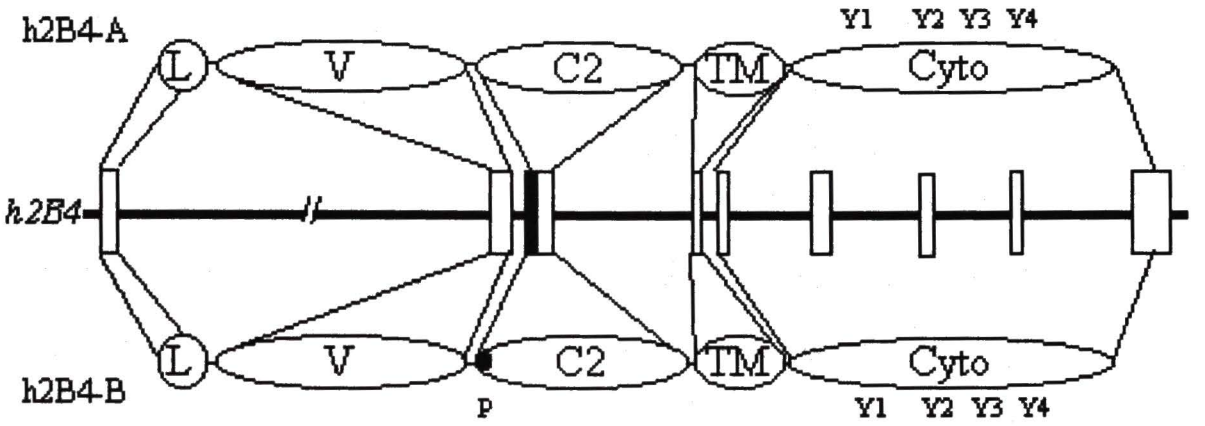
Figure 21. Genomic DNA blot analysis of CS1. Human genomic DNA (20 μg) from liver were digested with the restriction enzymes BamH I, EcoR I, Hind III, and Xba I, electrophoresed in 0.8% agarose, blotted, and hybridized with a ^{32}P labeled, full length CS1 cDNA probe. Sizes of DNA standards are shown at the left.


kb
BamH I
EcoR I
Hind III
Xba I
48
20
10
8
6
5
4
3
2

A blast search using the CS1 cDNA versus the genomic databases at Genbank identified a 196 kb contiguous sequence containing the *CS1* gene (Genbank accession number AL121958). Analysis of the complete gene sequence available in the contiguous sequence confirmed the size of CS1 and further revealed that the size discrepancy is due to a smaller first intron of 8.7 kb in *CS1* gene compared with a 17 kb first intron in *2B4* gene (Figure 22). The *CS1* gene structure extrapolated from the contiguous sequence reveals both the gene size and number of exons in *CS1* are smaller than *2B4*. Additionally, CS1 follows the same general exon arrangement with 3 exons coding for the extracellular domains, a single exon for the transmembrane domain, and only 3 exons for the cytoplasmic domain. The alteration that produces the short variant of CS1 reported by J. Murphy (19A24) is due to the elimination of exon 5 which leads to a frame shift immediately after the transmembrane domain, despite the inclusion of exons 6 and 7. Figure 22 demonstrates the exon usage for the various 2B4 and CS1 receptors.

Genes encoding members of the CD2 subset of receptors are located on human chromosome 1. *CD2* and *CD58* genes are located on the short arm of human chromosome 1 at 1p13 (Clayton et al., 1988; Sewell et al., 1988). *2B4, CD48, CD84, SLAM* and *Ly-9* reside on the long arm at 1q23-24 (Aversa et al., 1997a; de la Fuente et al., 1997; Sandrin et al., 1996; Staunton et al., 1989; Tangye et al., 1999). The contiguous sequence is located on the long arm of chromosome 1 at 1q23-24. Additionally, *CS1* is located in between *CD48* and *Ly-9* (Figure 23).

Figure 22. Schematic representation of the genomic organization of the exons and introns for the *CS1* gene and splice variants derived thereof. Exons are represented by rectangles. Protein domains of the splice variants are indicated by L, V, C2, TM, and Cyto and refer to leader sequence, Ig V-like domain, Ig C2-like domain, transmembrane domain, and cytoplasmic domain, respectively. Y1-Y2 represents tyrosine containing motifs.


h2B4-A
L
V
C2
TM
Y1 Y2 Y3 Y4
Cyto
h2B4
h2B4-B
L
V
C2
TM
Cyto
P
Y1 Y2 Y3 Y4

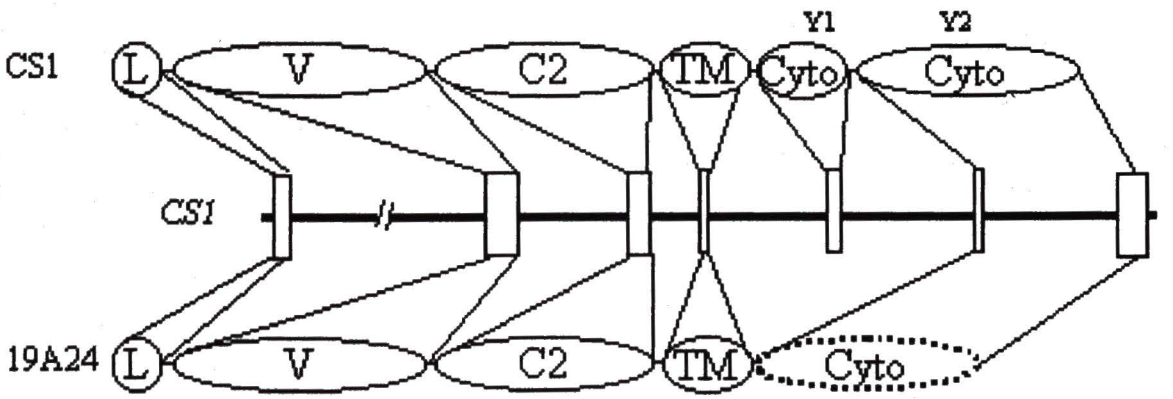
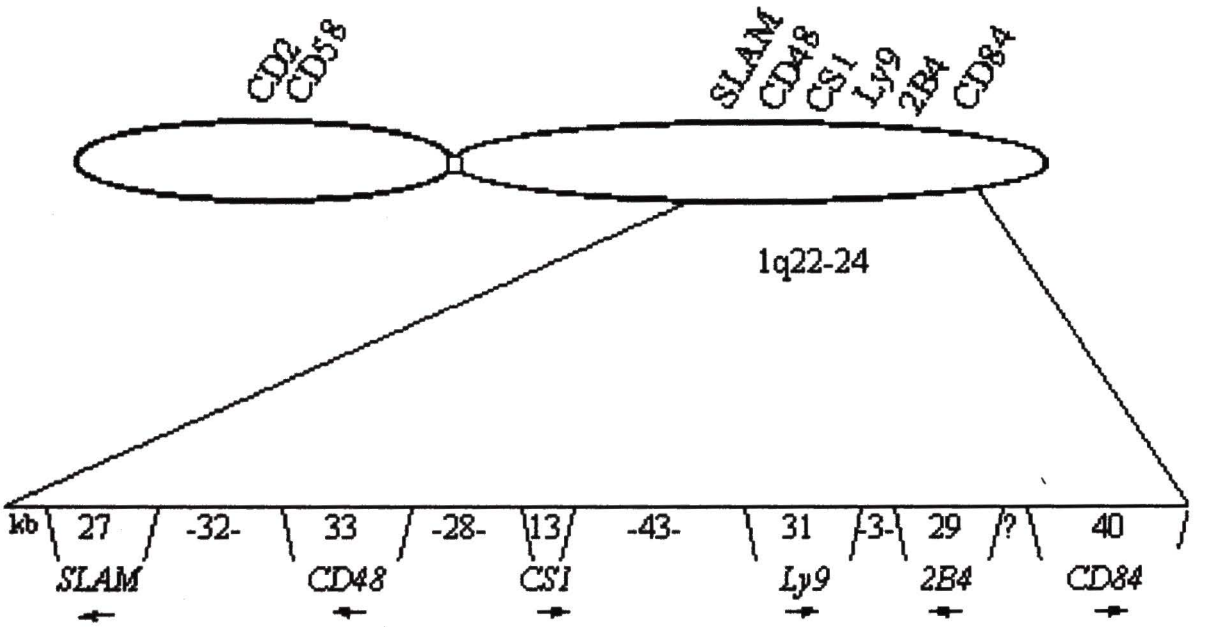

CS1
L
V
C2
TM
Y1
Cyto
Y2
Cyto
CS1
19A24
L
V
C2
TM
Cyto

Figure 23. Map of the CD2 subset of receptors located on human chromosome 1. *CD2* and *CD58* are located on the short arm of chromosome 1 at 1p13. *CS1* is located on the long arm of chromosome 1 at 1q23-24 along with *CD48, CD84, SLAM (CD150), Ly9 (CD229),* and *2B4 (CD244)*. The inset shows the gene sizes and spacing and is based on analysis of contiguous genomic sequences (Genbank accession numbers AL121985 and NT0044061). The relative position of the genes are listed in kilobases. Gene sizes and intra-gene spacing in kilobases (kb) are listed underneath. The arrows indicate relative gene orientation.


CD2
CD58
SLAM
CD48
CS1
Ly9
2B4
CD84
1q22-24
kb
27
-32-
33
-28-
13
-43-
31
-3-
29
?
40
SLAM
CD48
CS1
Ly9
2B4
CD84

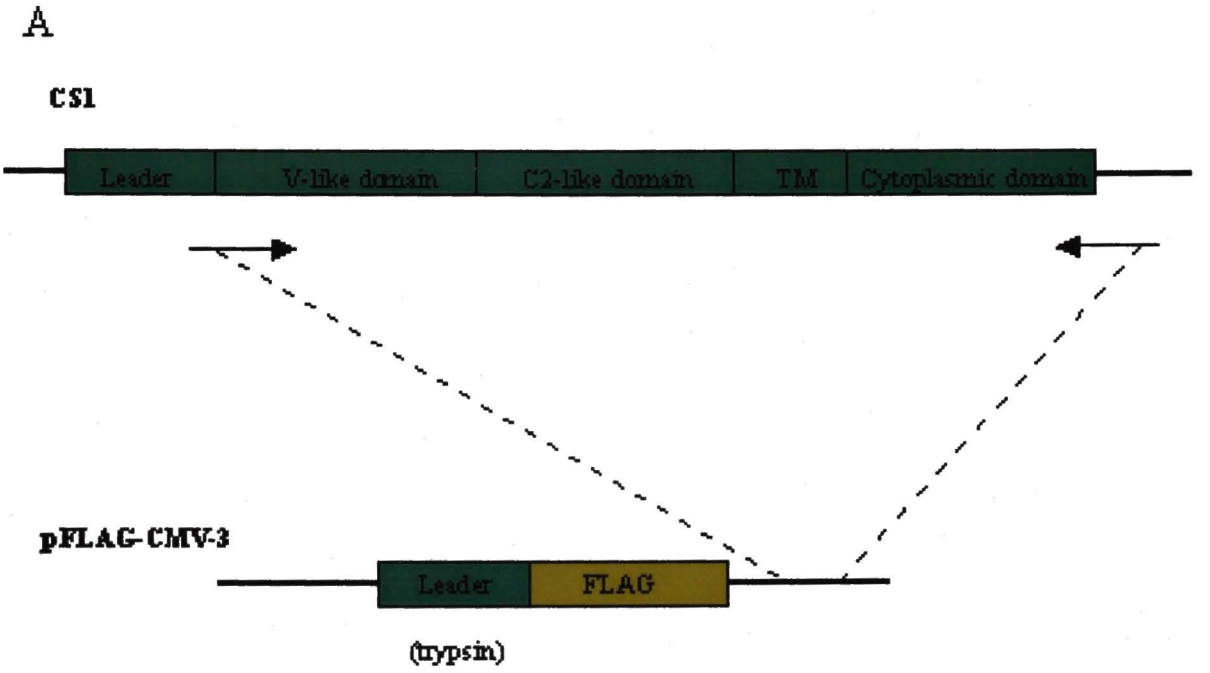
Assay for lytic activity

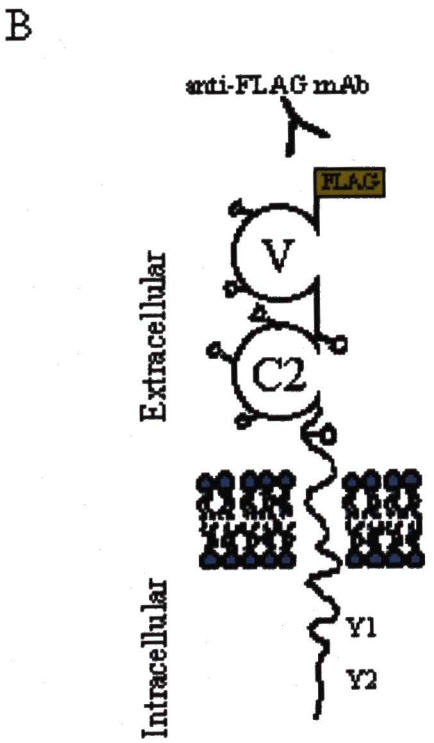
The human NK cell line YT alone or expressing the CS1-FLAG construct (Figure 24) was tested for the ability to lyse K562 and P815 target cells in a 4 hour ^{51}Cr release assay. C1.7 which binds 2B4 stimulated lysis of K562 target cells (Figure 25). Alternatively, FLAG antibody had no effect on YT or YT / CS1-FLAG cells. Furthermore, it did not modulate the lysis induced by C1.7. In a similar fashion, lysis of P815 cells in a redirected lysis assay was efficient upon 2B4 stimulation and FLAG treatment did not reveal and effect. The FLAG antibody may not bind the CS1 receptor construct in a similar fashion as the C1.7 stimualtion of 2B4. Alternatively, the CS1 receptor may not transduce signals in YT cells.

Interferon γ release assay

YT or YT expressing the CS1-FLAG construct were incubated in the presence of K562 cells and antibodies. YT cells performed as expected to 2B4 stimulation via the C1.7 mAb (Figure 26). Alternatively, the YT / CS1-FLAG expressing cells were generally non-productive of IFN-γ in response to treatment. Furthermore, they did not respond to the FLAG antibody despite the fact that it was able to surface label the cells. It appears that over-expression of the CS1 receptor may impair IFN-γ release in YT cells.

Figure 24. A construct to introduce a FLAG epitope into the N-terminal of the CS1 receptor A. Diagram of the CS1-FLAG construct. CS1 represents the cDNA. Arrows indicate the position of PCR primers used to amplify the cDNA without the leader sequence. pFLAG-CMV3 is the expression vector with a leader sequence (trypsin) and a FLAG epitope under the expression of a CMV promoter. The CS1 cDNA fragment was cloned in frame into the expression vector with EcoR I and Xba I sites that were introduced via the PCR primers. B. Diagram of the CS1 / FLAG construct as expressed on the cell surface of a transfected cell. C. Flow cytometry of CS1 / FLAG constructs expressed in the cancer cell lines, BW and YT.


A
CS1
Leader
V-like domain
C2-like domain
TM
Cytoplasmic domain
pFLAG-CMV-3
Leader
FLAG
(trypsin)


B
anti-FLAG mAb
FLAG
V
C2
Extracellular
Intracellular
Y1
Y2

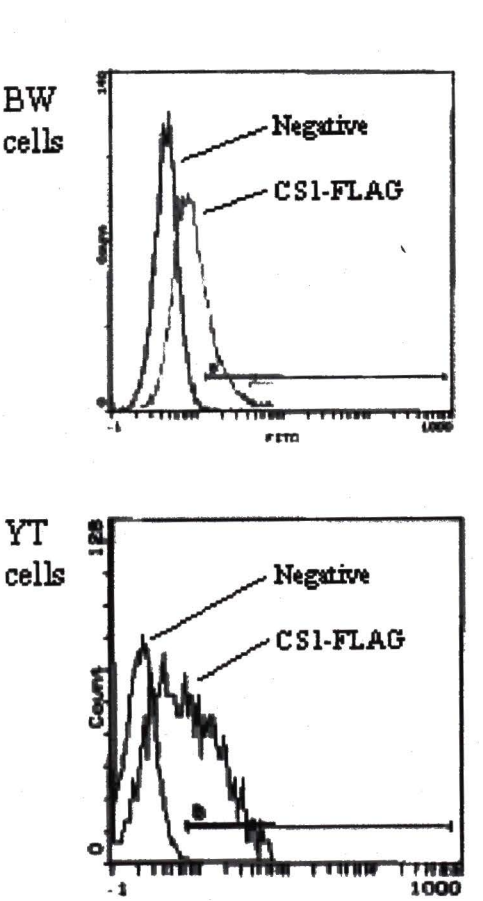

C
BW cells
Negative
CS1-FLAG
YT cells
Negative
CS1-FLAG
Count
0
128
-1
1000

Figure 25. Cytolytic activity of the human NK cell line YT or YT transduced with a CS1-FLAG construct against various targets in presence of antibodies. C1.7 is an anti-2B4 mAb which induces cytotoxicity. The FLAG antibody recognizes the FLAG epitope which was introduced into the extracellular domain of the CS1 receptor and expressed by YT cells in the YT / CS1-FLAG cells. Cytotoxicity was determined in a standard 4 hr ^{51}Cr release assay. K562 cells represent direct lysis by the YT cells and P815 demonstrate redirected lysis. The figure depicts a representation of three independent trials.

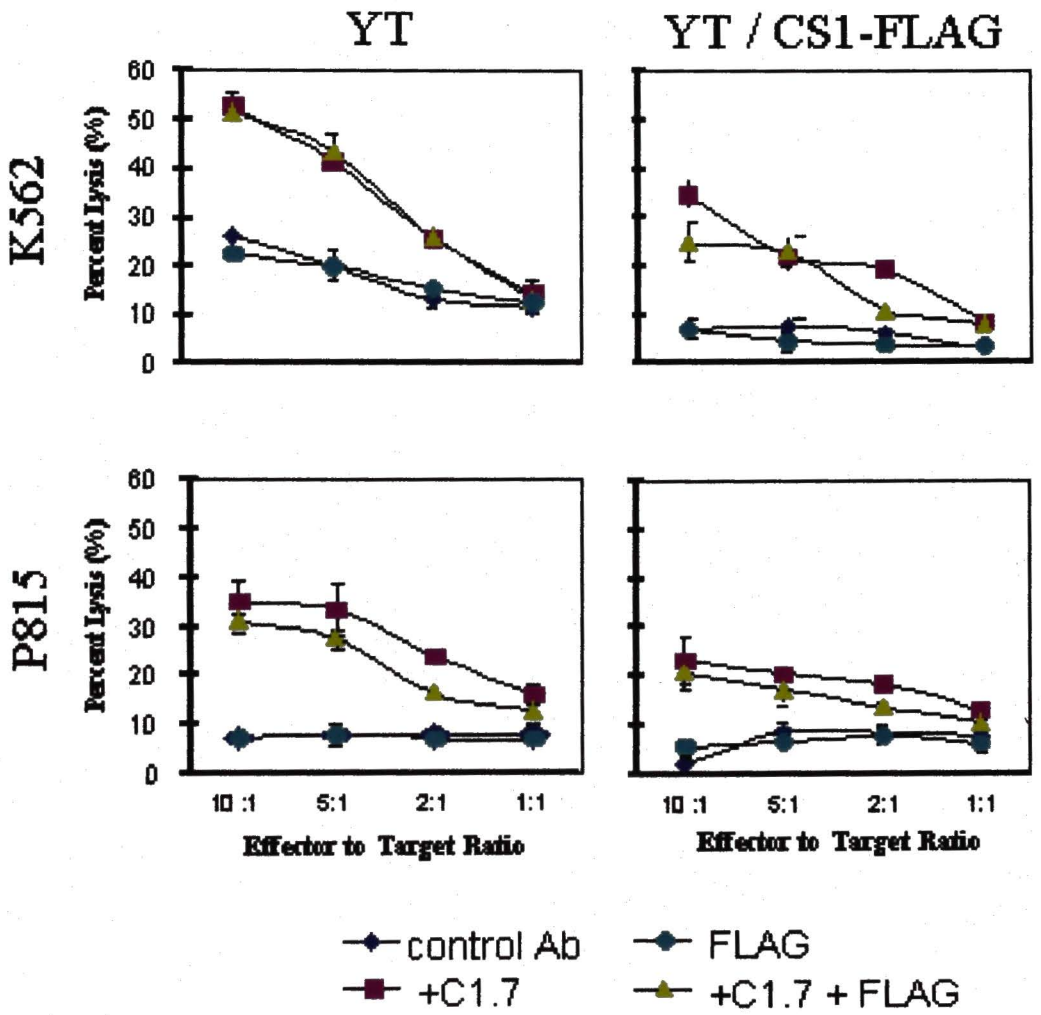
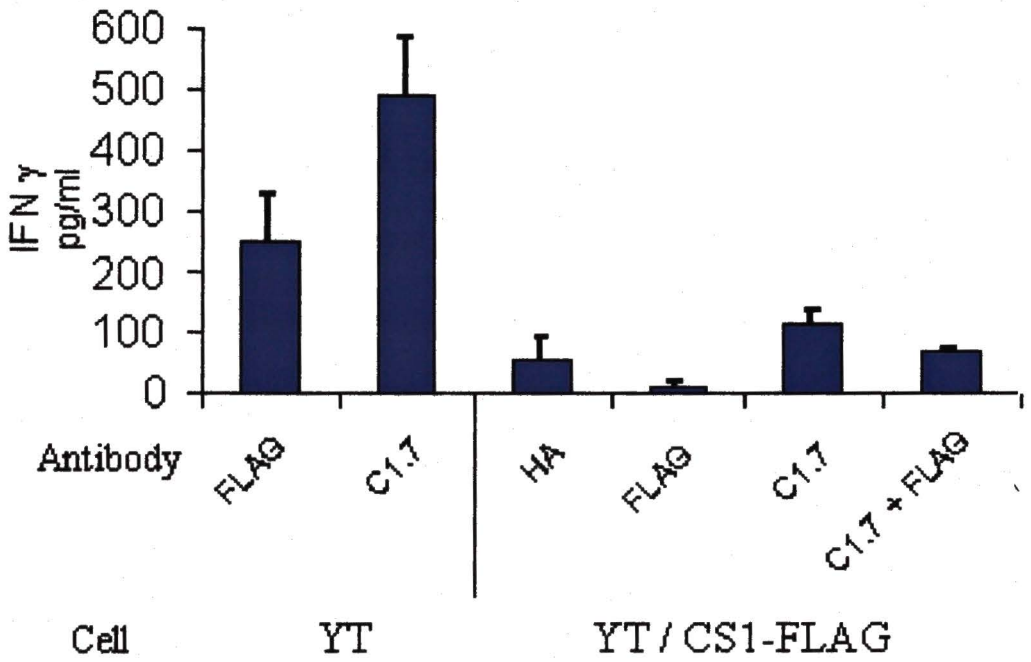

YT
YT / CS1-FLAG
K562
P815
Percent Lysis (%)
60
50
40
30
20
10
0
10 :1
5:1
2:1
1:1
Effector to Target Ratio
control Ab
+C1.7
FLAG
+C1.7 + FLAG

Figure 26. CS1-FLAG induced IFN-γ secretion in YT cells. YT cells or YT cells transduced with a CS1-FLAG construct were stimulated with various antibodies for 18 hours and IFN-γ was measured by ELISA. N=3.


600
500
400
300
200
100
0
IFN γ
pg/ml
Antibody
FLAG
C1.7
HA
FLAG
C1.7
C1.7 + FLAG
Cell
YT
YT / CS1-FLAG

CHAPTER 5

DISCUSSION

NK cell functions include the direct lysis of neoplastic cells and infected cells. Elucidation of NK cell target recognition is vital to understand the immune response. NK cells play a pivotal role in the innate immune response. They are the early responders to infection and protect the host by the production of IFN-γ. These early responses to infection are required since the adaptive immune response takes several days to respond to infection. NK cell function is regulated by signaling through surface receptors. Identification of the receptors and elucidation of their biology is required to understand NK cell regulation. This dissertation describes several studies to identify and characterize NK cell receptors. I identified three new members of the C-lectin superfamily and two new members of the CD2 subset of receptors.

Identification of novel C-type lectins expressed on NK cells

Chapter 2 describes the cloning and molecular characterization of two new members of the human NK gene complex. The conserved C-type CRD found in NK cells receptors localized to the NK gene complex allowed identification of related sequences in the EST database. The predicted peptides of LLT1 and LLT2 bears strong similarity to CD69 and AICL (Hamann, Fiebig and Strauss, 1993; Hamann et al., 1997; Lopez-Cabrera et al., 1993; Ziegler et al., 1993). The function of CD69 has been extensively

characterized. Cross-linking with anti-CD69 Ab activates the cell specific functions of lymphocytes, granulocytes, monocytes, and platelets (Testi et al., 1994). Furthermore, CD69 is a useful indicator of cell activation and immune arousal. The signaling of neither AICL nor the LLTs has been studied yet. In light of the structural similarity of CD69 to AICL and the LLTs, it is likely these new transcripts will demonstrate novel functions.

The observation of multiple bands in Northern analysis implies the existence of highly related transcripts or splice variants of LLT1. These could be LLT2 transcripts or other receptors. Both LLT1 and CD69 are expressed in lymphocytes, at a high level in NK cells and less in T and B cells (Hamann, Fiebig and Strauss, 1993). We do not know whether LLT1 may be inducible on T and B cells. The restricted expression of LLT1 to tissues representing the later stages of NK cell differentiation implicates it as a receptor involved in immune response rather than development. The Southern blot showed a simple pattern indicating a single gene or a small number of genes. A similar pattern has been reported for *CD69* (Santis et al., 1994) and *AICL* (Hamann et al., 1997). The *CD69* gene is localized to approximately 15 kb and the *AICL* gene is approximately 20 kb. Taken together, this supports that *LLT1* may be a single gene..

We co-localized the *LLT1* gene to within 100 kb of the *CD69* gene. The *AICL* gene has been previously localized to 0.3 cM proximal to the *CD69* gene (Hamann et al., 1997). Consequently, the proximity of the genes and the sequence similarities between the AICL, CD69, and LLT1 cDNAs suggests that the genes might be derived from the duplication of a common ancestral gene.

In conclusion, sequence analysis and chromosomal localization classify LLT1 and LLT2 as new members of the NK cell receptors located in the human NK gene complex. Sequence similarity to CD69 and AICL suggest that LLT1 and LLT2 may have a comparable role in the immune system. Antibodies that recognize the LLT receptors will allow the elucidation of the functional role of the LLTs in human lymphocytes.

Identification of novel members of the CD2 subset of receptors

2B4 appears to act as a primary modulator of activation signal in murine NK and γδ T cells (Garni-Wagner et al., 1993; Schuhmachers et al., 1995a; Schuhmachers et al., 1995b). Alternatively, it may act as a costimulatory molecule in lymphocytes as indicated by CD3 and 2B4 stimulation (Schuhmachers et al., 1995b). Similarly, anti-SLAM as well as anti-CD48 mAb both show costimulatory effects on B cell activation (Klyushnenkova et al., 1996; Punnonen et al., 1997). I describe the cloning and characterization the human homologue of 2B4 in chapter 3. Its role in the human immune response has proven to be extensive and of great significance as described below.

2B4 is a member of the Ig superfamily and further classified as a member of the CD2 subfamily. Murine 2B4 is expressed as two alternately spliced variants from a single gene. The human homologue is most similar to the long splice variant of murine 2B4 and may be the only form expressed in the human. This is supported by the appearance of only one band in poly(A)$^+$ RNA in Northern analysis. Human 2B4 appears to be widely expressed as indicated by Northern blot analysis of NK and T cell lines.

Sequence alignment of human 2B4 with other members of the CD2 subfamily indicates that the Ig-like domains are well conserved and the overall similarity is greatest between 2B4, SLAM (CDw150), and CD84 in that they all share additional features in their cytoplasmic domains (Aversa et al., 1997b; Cocks et al., 1995; Davis and van der Merwe, 1996; de la Fuente et al., 1997). Human 2B4 contains six tyrosines in its cytoplasmic tail, four of which are embedded in a motif (TxYxxI/V) that is likely to be phosphorylated by group I kinases (Zhou and Cantley, 1995a) and interact with SH2 domains (Kuriyan and Cowburn, 1997). These four tyrosine motifs are conserved in the long form of murine 2B4 and are similar to motifs found in the cytoplasmic domains of SLAM and CD84 (Cocks et al., 1995; de la Fuente et al., 1997). Interestingly, one of these motifs in SLAM has been shown to interact with the SH2 domain of SLAM associated protein (SAP), the recently identified X-linked lymphoproliferative disease (XLP) gene (Sayos et al., 1998). The region between the transmembrane domain and the membrane proximal tyrosine is highly conserved in 2B4, SLAM, and CD84 suggesting that several members of the CD2 subfamily may interact with the newly identified SAP molecule or related molecules containing SH2 domains.

The interaction of the SAP molecule with multiple receptors of the CD2 subset and the variety of immune dysregulation seen in the XLP disease led me to postulate that there may be additional receptors of the CD2 subset that contain the novel tyrosine motif in their cytoplasmic domains. Chapter 4 describes the cloning and characterization of one such receptor, CS1.

The 2B4 and CS1 receptors belong to the CD2 subset of the Ig superfamily due to homology in its extracellular domains (Davis et al., 1998). The other members of the CD2 subset are: CD2, CD48, CD58, CD84, Ly9, and SLAM (Tangye, Phillips and Lanier, 2000). All family members contain at least one membrane distal Ig V-like domain and an Ig-C2-like domain. The exception to the two Ig-like domains of the family members is the Ly9 (CD229) molecule. It has four Ig-like domains appearing as a duplication of the extracellular V and C2-like domains of the other CD2 subset receptors. Each receptor has conserved cysteine residues in the C2-like domain that form disulfide bonds (Boles and Mathew, 2001; Boles et al., 1999b; Castro et al., 1999; Cocks et al., 1995; de la Fuente et al., 1997; de la Fuente et al., 1999; Mathew et al., 1993; Sandrin et al., 1992; Sandrin et al., 1996; Seed, 1987; Seed and Aruffo, 1987; Tangye et al., 1999). Additionally, all of the family members have an N-terminal extracellular domain, a single transmembrane domain, and a cytoplasmic tail with the exception of CD48, which is linked to the cell surface membrane by glycosyl phosphotidyl inositol (GPI) (Staunton et al., 1989). Alternatively, CD58 can exist with either a short transmembrane domain or a GPI-linkage (Seed, 1987).

The variability of the cytoplasmic domains within the CD2 subset suggests the basis of their functional differences. The GPI-linkage of CD48 and CD58 allows them to function as ligands and contribute to signaling (Moran and Miceli, 1998; Stefanova et al., 1991). The cytoplasmic domain of CD2 is unique to the CD2 subset as it contains novel proline-rich segments (PPPGHR) that are essential to its binding of the CD2 binding protein 2 (CD2BP2) (Dustin et al., 1998; Nishizawa et al., 1998). Furthermore, proline

residues in the CD2 cytoplasmic domain have been shown to regulate T cell adhesion and activate kinase activity such as phosphoinositide 3 (PI3)-kinase and the Tec-family tyrosine kinase ITK (King et al., 1998; Kivens et al., 1998). The cytoplasmic domains of 2B4, CS1, SLAM, CD84, and Ly9 all contain at least two instances of a novel tyrosine motif (TxYxxV/I) (Boles and Mathew, 2001; Boles et al., 1999b; Castro et al., 1999; Cocks et al., 1995; de la Fuente et al., 1997; de la Fuente et al., 1999; Mathew et al., 1993; Sandrin et al., 1992; Sandrin et al., 1996; Tangye et al., 1999). The similarity of the novel tyrosine motif to an immuno-tyrosine-inhibiting-motif (ITIM) suggests that it may play a role in modulating protein interactions (Bakker et al., 2000b; Blery, Olcese and Vivier, 2000).

The exon arrangement for the *2B4* and *CS1* is consistent with other CD2 subset members (Castro et al., 1999; Diamond et al., 1988; Sandrin et al., 1992; Sandrin et al., 1996; Wong et al., 1990) and consists of an exon per domain for the leader sequence, V-like domain, C2-like domain, and transmembrane domains. Multiple exons code for the intracellular regions (Boles and Mathew, 2001; Kumaresan and Mathew, 2000; Stepp et al., 1999b) (*Fig 3*). Furthermore, differential exon usage leads to splice variants of the receptors.

Understanding the functional relevance due to the variation between the cytoplasmic domains between receptors is further complicated by the existence of the splice variations within a receptor. Both murine 2B4 and SLAM demonstrate splice variants that alter the number of novel tyrosine motifs within the cytoplasmic domains from 4 and 2 to only 1 for each, respectively (Castro et al., 1999; Mathew et al., 1993;

Stepp et al., 1999b). The short variant of the SLAM receptor has been observed in the human whereas the 2B4 short clone has not been detected (Cocks et al., 1995). Additionally, both CD84 and CS1 (19A24 submitted by J. J. Murphy) exist in isoforms that completely eliminate the two novel tyrosine motifs present in the long form of each molecule, Genbank accession numbers U96627 and AJ271869, respectively. An additional splice variant for the murine Ly108 has also been reported (Peck and Ruley, 2000). The two molecules only demonstrate a change in the cytoplasmic domain that does not alter the number of tyrosine motifs.

The murine *2B4* gene consists of at least 9 exons with one exon dedicated to the leader sequence, V-like, C2-like, and transmembrane domains, each. The first intron is rather large at approximately 16 kb giving rise to a total gene size of about 27 kb. Variable exon usage gives rise to the two forms of 2B4 in the mouse (Stepp et al., 1999b). 4 exons encode the 2B4-L form's cytoplasmic domain, giving rise to 4 tyrosine motifs. 2B4-S is identical to the 5' end of 2B4-L, differing only at the 3' end in a portion of the cytoplasmic domain and the 3' untranslated sequence. 2B4-S is the product of the same first 5 exons in 2B4-L with the usage of a novel exon at the C-terminal.

Based on sequence comparison, the human 2B4 cDNA I cloned was homologous to the m2B4-L form. Subsequently, a second transcript of 2B4 (h2B4-B) has been identified with an additional 15 nucleotides inserted at the junction between the V-like and C2-like domains. Otherwise, the transcript was identical to the previously reported clone (Kumaresan and Mathew, 2000). Characterization of the human *2B4* gene revealed a similar structure to the mouse gene. The human 2B4 gene contains nine exons, the first

four exons encode the leader sequence, V-like, C2-like, and transmembrane domains and the remaining five exons encode the cytoplasmic domain. Furthermore, the first intron is also very large at approximately 17 kb. The difference between h2B4-A and h2B4-B arises from differential splicing of exon 3. Exon 3 contains an internal splice site and the first 15 nucleotides of exon 3 are spliced out of h2B4-A. h2B4-B contains the 15 nucleotides which results in the additional 5 amino acids, including a proline residue. The difference in the extracellular domains of these two receptors might mediate differential ligand interaction or affinity. Because the intracellular domains are identical, they most likely deliver similar signals.

The *CS1* gene structure can also be extrapolated from a contiguous gene sequence produced by the human genome project (Genbank accession number AL121958). Both the gene size and number of exons in *CS1* are smaller than *2B4* although the exon usage pattern is generally the same. *CS1* is approximately 13-14 kb as estimated by Southern analysis (unpublished observation) and sequence analysis, compared to 26-30 kb for murine and human *2B4* genes. The size discrepancy is due to a smaller first intron of 8.7 kb in the *CS1* gene compared with a 17 kb first intron in the *2B4* gene. Additionally, CS1 follows the same exon arrangement with 3 exons coding for the extracellular domains, a single exon for the transmembrane domain, and only 3 exons for the cytoplasmic domain. The alteration that produces the short variant of CS1 (19A24) is due to the elimination of exon 5 which leads to a frame shift immediately after the transmembrane domain, despite the inclusion of exons 6 and 7. Figure 3 demonstrates the exon usage for the various 2B4 and CS1 receptors.

Although, the existence of splice variants is intriguing in light of their potential differences in signaling, there is no direct biochemical evidence to support their expression. *In vitro* analysis of the m2B4 variants suggests potential signaling differences. Transfection of the murine 2B4 variants and mutants thereof into the rat NK cell line, RNK-16 allows their function to be analyzed. Interestingly, the two forms of 2B4 had opposing functions (Schatzle et al., 1999). 2B4-S was able to mediate redirected lysis of P815 tumor targets, suggesting that this form represents a constitutively activating receptor. However, 2B4-L expression led to an inhibition of redirected lysis of P815 targets. In addition, 2B4-L inhibited lysis of YAC-1 tumor targets. Removal of the novel tyrosine motifs abrogates the inhibitory function of 2B4-L. Like other potentially inhibitory receptors, 2B4-L associated with the tyrosine phosphatase SHP- 2. These results suggest potentially different roles for the two isoforms. Modulation of the 2B4 pathway by other adaptor molecules probably regulates the ability of 2B4 to send different types of signals.

Cellular immune responses have been determined for some members of the CD2 subset. CD48 and CD58 function as ligands and their GPI-linkages probably allow them to aggregate lipid rafts when they are cross-linked (Moran and Miceli, 1998; Stefanova et al., 1991). Cross-linking of CD48 on T cells can deliver a T cell receptor (TCR) co-stimulating signal (Flament et al., 1996; Latchman and Reiser, 1998; Moran and Miceli, 1998). Cross-linking CD48 on B cells can deliver a CD40 co-stimulating signal (Klyushnenkova et al., 1996) and T cell independent help for differentiation (Hoffmann et al., 1998). Blocking CD48 *in vivo* or *in vitro* suppresses cytotoxic T lymphocyte (CTL)

induction and NK cell activation (Chavin et al., 1994), but this may be due to a lack of 2B4 signaling.

CD2 is implicated as an important costimulatory molecule in lymphocyte activation. Ca^{2+} flux and tyrosine phosphorylation occur following CD2 stimulation resulting in proliferation, cytotoxicity, and IFN-γ and IL-2 release (Holter et al., 1996; Meuer et al., 1984; Siliciano et al., 1985). Furthermore, proline residues in the CD2 cytoplasmic domain have been shown to activate kinase activity such as phosphoinositide 3 (PI3)-kinase and the Tec-family tyrosine kinase ITK (King et al., 1998; Kivens et al., 1998). Additionally, adhesion of T cells to antigen presenting cells (APC) or NK cells is increased following CD2 signaling (Bierer and Hahn, 1993; Holter et al., 1996).

Despite the functions of the CD2 molecule that have been demonstrated, its requirement for immune function has not been demonstrated. Experimental data on CD48 bears reinterpretation due to the differences observed in the phenotypes of the CD2 deficient mouse (Killeen, Stuart and Littman, 1992) and the CD48 deficient mouse (Gonzalez-Cabrero et al., 1999). CD2 deficient mice have a very mild phenotype, whereas CD48 deficient mice have significant defects in $CD4^+$ T cells (the function of other lymphocyte subsets was not addressed). Another example is the enhanced survival of cardiac tissue grafts in mice treated with blocking mAb to CD48 and CD2 as compared to mAb to CD2 alone (Qin et al., 1994). These perplexing results can now be explained by the presence of an alternative ligand (such as 2B4) for CD48. This suggests that the alternative interaction of CD48 and 2B4 is crucial for immune function. This still needs

to be confirmed by the phenotype of 2B4 deficient mice. One would expect their immune responses to mirror the CD48 knock out mice.

SLAM mAbs promote the TCR dependent proliferation of human T cells, independently of CD28 (Aversa et al., 1997a; Cocks et al., 1995). Signaling via SLAM was also particularly effective at inducing IFN-γ secretion by T cells, including $CD4^+$ human Th1 cells and had little effect on the secretion of cytokines characteristic of a Th2 phenotype (Carballido et al., 1997; Castro et al., 1999; Cocks et al., 1995). Thus, activation via SLAM may contribute to the Th1 type cellular immune response via cytokines produced by activated T cells.

Only recently has the human homologue of 2B4 been identified. I initially performed a northern blot with the murine cDNA versus mRNA from human NK cells. No definitive bands were observed. Alternatively, Southern analysis of human genomic DNA with the murine cDNA did show distinct bands. Therefore, I screened a genomic DNA library with mouse 2B4 and obtained sequences from coding regions of the *2B4* gene. I subsequently screened a human NK cell cDNA library and isolated a cDNA encoding human 2B4 (Boles et al., 1999b). Several other labs also reported cloning of h2B4 by cDNA screening using the mouse 2B4 cDNA in addition to expression cloning using an anti-2B4 mAb, C1.7 (Kubin et al., 1999; Tangye et al., 1999).

Studies of 2B4 function in the human immune system have been facilitated by the generation of three different monoclonal antibodies that bind human 2B4. The first of these is C1.7 (Valiante and Trinchieri, 1993). Hybridomas were generated with spleen cells from BALB/c mice that had been immunized with cultured human NK cells. The

hybridomas were screened for increased susceptibility to killing by NK effector cells in a ^{51}Cr release assay. In flow cytometric analysis of human peripheral blood leukocytes (PBL), C1.7 stained virtually all NK cells, about 50% of $CD8^+$ T cells, about 75% of peripheral γδ T cells. Monocytes also stained positively with mAb C1.7, although with a lower intensity than lymphocytes. A single 38 kDa protein was seen by western blotting whole cell lysates of cultured NK cells with C1.7. Almost all of the non-MHC-restricted cytotoxic activity of IL-2 cultured $CD8^+$ T cells belonged to the $C1.7^+$ population. This was true using both tumor cell lines and virally infected target cells.

With both fresh and cultured NK cells, C1.7 mAb mediated the redirected lysis of an FcR^+ target cell. However, C1.7 mAb (soluble, immobilized on plastic or presented by FcR^+ cells) could not induce NK cell granule release as measured by serine-esterase activity. C1.7 mAb treatment of NK cells did induce cytokine production, but only when C1.7 mAb was presented by FcR^+ cells, neither soluble nor plastic immobilized mAb was active. C1.7-dependent production of IFN-γ required the presence of IL-2, IL-12 or target cells (K562), whereas C1.7-dependent production of IL-8 was independent of added cytokines (Johnson, Goldfarb and Mathew, 2000; Valiante and Trinchieri, 1993). No production of tumor necrosis factor-α (TNF-α) was observed under any of these conditions. Soluble C1.7 enhanced the proliferation of fresh PBL cultured for 6 days in the presence of IL-12 and IL-2. However, soluble C1.7 inhibited proliferation of purified NK cells cultured for seven days. Cross-linking C1.7 on purified cultured NK cells with goat-anti-mouse-Ig $F(ab')_2$ induced Ca^{2+} signaling and initiated polyphosphoinositol

turnover. Matrix metalloproteinases (MMP) are involved in NK cell invasiveness via the degradation of extracellular matrix (Albertsson et al., 2000; Kim et al., 2000). C1.7 stimulates the expression of MMP-2 (Chuang et al., 2000). Therefore, C1.7 mAb demonstrates that in addition to modulating cytolytic function and cytokine production of NK cells, activation through surface 2B4 may play a role in up regulating the machinery for degradation of extracellular matrices to promote invasion of tumors by NK cells.

The second mAb that binds human 2B4 was obtained by generating mAb against the human NK cell line YT. mAb 158 induced YT and NK cell clones (but not T cell clones) to kill the FcR^+ target P815 by redirected lysis (Boles et al., 1999b; Nakajima et al., 1999). Cross-linking 2B4 with mAb 158 did not induce cytokine production or proliferation by bulk LAK cultures (T and NK cells) or by monocytes. In a redirected killing assay, mAb 158 enhanced NK cell-mediated killing induced with suboptimal anti-CD16 stimulation, but had no affect on T cell-mediated killing induced with suboptimal anti-CD3 stimulation.

The third mAb that binds human 2B4 is PP35 (Moretta et al., 1992). When PP35 was used in a redirected killing assay of the murine FcR^+ target, P815, the enhancement of killing by $2B4^+$ human NK cell clones was heterogeneous and depended on the expression of NKp46 by the NK cell clone (Sivori et al., 2000). Redirected lysis was triggered by the 2B4 mAb PP35 only for $NKp46^{bright}$ NK cell clones. This was interpreted to be due to the expression by the murine target cell of the NKp46 ligand, and a co-stimulating function for 2B4. The interpretation of a co-stimulating role for 2B4 was

supported by the demonstration that 2B4 mAb PP35 co-stimulated anti-NKp44 or anti-CD16-mediated killing by NKp46^{dull} NK clones in a redirected killing assay.

Finally, CD48 stimulates NK cell function. Transfection of target cells with the 2B4 ligand, CD48, enhanced NK cell-mediated killing of some targets, but not others. Where target cell expression of CD48 enhanced the NK cell mediated killing, blocking of CD48 binding to 2B4 with CD48 mAb completely blocked the enhanced killing, whereas mAb 158 partially blocked the enhanced killing (Nakajima et al., 1999). Additionally, soluble 2B4 and CD48 recombinant proteins have been used to study the function and interaction of human 2B4 with CD48 (Kubin et al., 1999). Recombinant CD48 protein immobilized on plastic enhanced the killing of both Daudi and Raji targets by purified human NK cells. Stimulation of NK cells with immobilized CD48 alone did not induce IFN-γ production. However, in the presence of IL-2, IL-12, or IL-15 immobilized CD48 enhanced IFN-γ production approximately 3-fold. The requirement for anti-2B4 antibody plus further stimulation to produce IFN-γ was also observed with the C1.7 mAb against YT cells (Johnson, Goldfarb and Mathew, 2000). C1.7 alone did not produce a significant increase in IFN-γ release, but the addition of either IL-2 or co-incubation with K562 cells augmented the IFN-γ production.

Recombinant 2B4 protein immobilized on plastic co-stimulated the IL-4- or CD40L-dependent proliferation by purified peripheral blood B cells CD48 (Kubin et al., 1999). Furthermore, soluble 2B4 protein induced IL-12p40 and TNF-production by dendritic cells. These results suggest that 2B4 may modulate the function of CD48^{+} B

cells and dendritic cells as well as $2B4^+$ lymphocytes . Antibody treatment of T cells with mAb 158 did not result in any significant effects on function or proliferation (Nakajima et al., 1999). A similar result occurred in monocytes as well. The lack of response to 2B4 stimulation in T cells is intriguing considering the co-expression of SLAM in these cells. This suggests that cross-linking a receptor with the novel tyrosine motif is not the minimum requirement in T cell signaling. Whether the phenotype of $2B4^+$ T cells is different from those cells that respond to SLAM mAb or the signaling mechanism between the two receptors is significantly different, despite the similarities in their cytoplasmic domains, remains to be demonstrated. Immune system development and function in 2B4 deficient mice will probably reveal the role of 2B4 in non-NK cells.

Many activating and inhibiting cell surface receptors involved in immune system function require specific tyrosine containing cytoplasmic sequences (referred to as tyrosine motifs) for their function. Phosphorylation of the tyrosine embedded within the motif sequence permits binding to src homology 2 (SH2) domains, while the amino acid sequence surrounding the tyrosine controls the specificity of SH2 domain binding. SH2 domains are found in a variety of kinases, phosphatases and adapter proteins involved in signaling cascades (Kuriyan and Cowburn, 1997).

Two well known lymphocyte receptor tyrosine motifs are the immuno-tyrosine-activating-motif (ITAM), D/ExxYxxL/Ix(6-8)YxxL/I and the immuno-tyrosine-inhibiting-motif (ITIM), I/VxYxxL/V. Many activating receptors non-covalently associate with ITAM-containing adapter proteins via a transmembrane charged amino acid. Activating receptors in this category include CD16, NKp46, NKp30, NKp44 and

those in the Ly-49, KIR, and CD94/NKG2 families. Three ITAM-containing adapter proteins are differentially utilized (Moretta et al., 2000; Tomasello et al., 2000b). DAP12 associates with activating members of the Ly-49, KIR, and CD94/NKG2 receptor families, as well as with NKp44. Receptor NKp30 uses the adapter CD3ζ while the activating receptor of the NKRP1 family uses the adapter FcεRIγ. The receptors NKp46 and FcγRIII use adapters CD3ζ and/or FcεRIγ. Cross-linking of ITAM-containing receptor complexes induces tyrosine phosphorylation of the ITAM and activation of the ZAP-70 / syk kinase signal cascade, reminiscent of TCR signaling via the CD3 ITAMs. The ITIM interacts with the SH2 domains of SHP-1, SHP-2 and/or SHIP phosphatases (Long, 1999). Most of the ITIM-containing receptors are involved in MHC-Class I recognition including the Ly49, killer cell Ig-like receptors (KIR), CD94/NKG2 complexes, and the immunoglobulin-like transcripts (ILT) / monocyte immunoglobulin-like receptors (MIR) families. Additionally, some of the paired immunoglobulin receptors (PIR) contain ITIMs, but their ligands have not been identified (Bakker et al., 2000b).

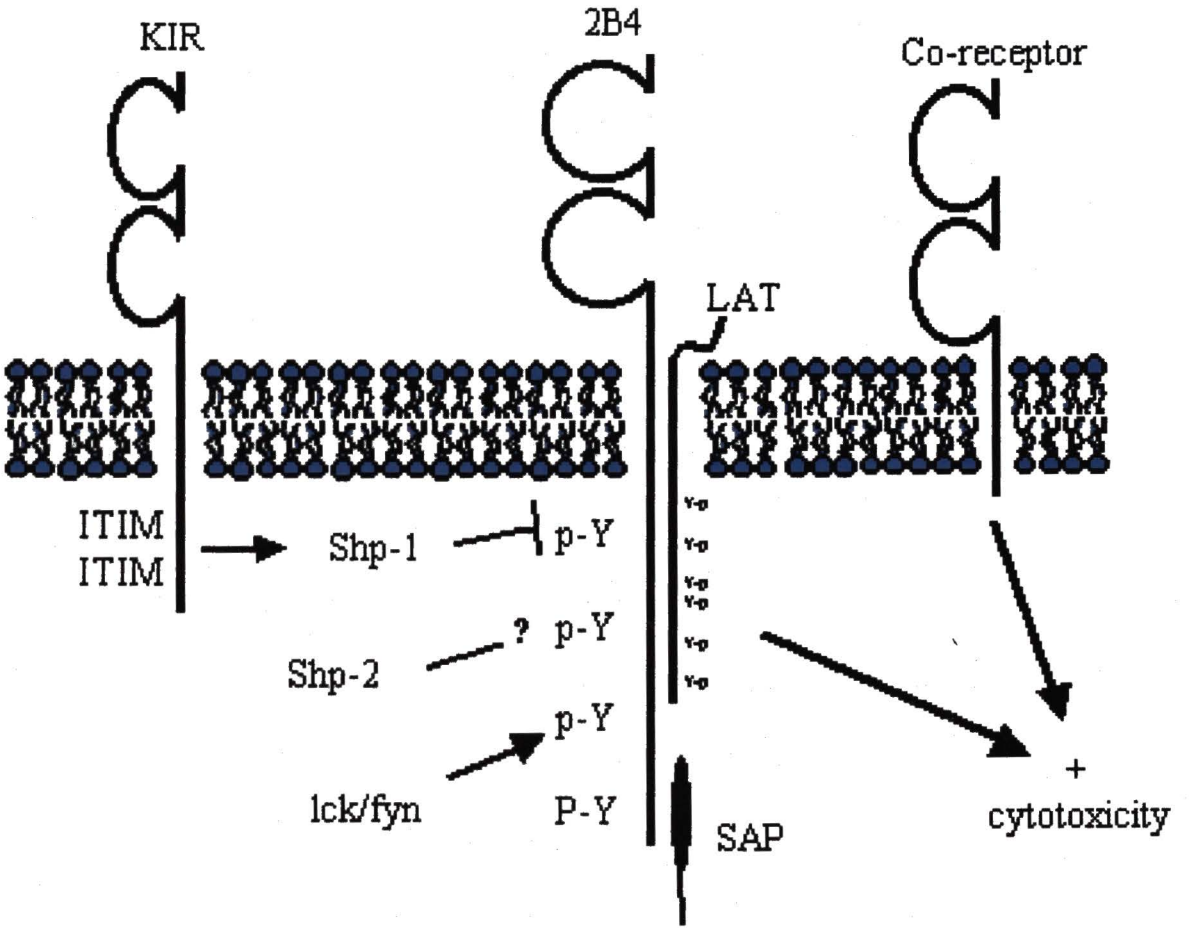
The cytoplasmic sequence of both murine and human 2B4 contains the repeated and conserved motif, TxYxxI/V (Boles et al., 1999b; Garni-Wagner et al., 1993). This tyrosine motif is expected to be an excellent target for Lck phosphorylation (Zhou and Cantley, 1995b). Although the 2B4 consensus tyrosine motif is neither an ITAM nor an ITIM, it is found on other receptors, including the related CS1, Ly9, CD84 and SLAM, all members of the CD2 subset of receptors.

Original characterization of the SLAM receptor by *in vitro* kinase assays revealed a protein kinase activity associated with it that is maintained even in Nonidet P- 40

lysates (Sidorenko and Clark, 1993). Subsequently, both Src-family kinases, Fgr and Fyn have been shown to interact with phosphorylated tyrosines in SLAM's cytoplasmic tail (Figure 27) (Mikhalap et al., 1999). Furthermore, Fyn and p56lck have been shown to interact with the 2B4 cytoplasmic domain (Nakajima et al., 2000; Sayos et al., 2000). Lastly, 2B4 tyrosine phosphorylation was greatly reduced in a Lck-deficient Jurkat cell line.

Both murine SLAM and 2B4 , upon tyrosine phosphorylation, associate with the src homology 2-domain-containing protein tyrosine phosphatase SHP-2, but not SHP-1 (Castro et al., 1999; Schatzle et al., 1999). Additionally, both human SLAM and 2B4 have been reported to interact with SHP-2, which can be excluded by the SAP molecule (Sayos et al., 1998; Tangye et al., 1999). Alternatively, no SHP-1 interaction was detected. These studies were performed in cells other than NK cells. In contrast, Parolini et al. observed that 2B4 was found to associate with SHP-1 and not SHP-2 in primary NK cell isolates (Parolini et al., 2000). Finally, Nakajima et al. reported that 2B4 did not associate with either SHP-1 or SHP-2 in the NK cell line, YT (Nakajima et al., 2000). Furthermore, they observed 2B4 interaction with SHP-2 only in transfected cells over-expressing 2B4, but not in normal or SAP-deficient NK cells.

Figure 27. 2B4 associates with several signaling molecules. Diagram of the various intracellular adaptor molecules that interact with the novel tyrosine motifs in the 2B4 cytoplasmic domain. ITIM represents immunotyrosine based inhibitory motif and p-Y represents phosphorylated tyrosines.


KIR
2B4
Co-receptor
LAT
ITIM
ITIM
Shp-1
p-Y
Shp-2
?
p-Y
p-Y
lck/fyn
P-Y
SAP
+
cytotoxicity

Co-ligation of ITIM- and ITAM-containing receptor complexes induces phosphorylation of ITIM tyrosine residues and activation of the tyrosine phosphatase, SHP-1. Activated SHP-1 blocks ITAM dependent activating signals. 2B4 signaling has been shown to be inhibited by the recruitment of SHP-1 via ITIM containing CD94/NKG2 or KIR (Figure 27) (Tangye, Phillips and Lanier, 2000; Watzl, Stebbins and Long, 2000). Whether the SHP-1 enzyme is acting directly upon the 2B4 cytoplasmic domain or on downstream molecules in the signal transduction pathway is unknown.

Phospholipase Cγ2 (PLCγ2) deficient mice show a reduced capacity for the lysis of YAC-1 targets when stimulated with anti-2B4 mAb (Wang et al., 2000). This suggests that 2B4 signaling may be dependent on PLC activation and the incomplete loss of lysis in the PLCγ2 deficient mice may be due to functional overlap with PLCγ1. Additionally, this suggests some similarity to the signaling pathway induced by TCR stimulation which utilizes the linker for activated T cells (LAT) (Tomlinson, Lin and Weiss, 2000). The 2B4 receptor is associated with LAT through immunoprecipitation (Figure 27) (Klem et al., 2000). Furthermore, 2B4 is constitutively localized to the glycolipid-enriched microdomains (GEMs) and its interaction is independent of phosphorylation or the presence of the 2B4 cytoplasmic domain (Klem et al., 2000). Further corroboration for the role of LAT in NK cell activation is that LAT becomes phosphorylated during natural cytotoxicity mediated by NK cells (Jevremovic et al., 1999). Recently, Bottino et al. definitively demonstrated that LAT is involved in 2B4 signaling (Bottino et al., 2000). They observed that 2B4 stimulation resulted in tyrosine phosphorylation not only of 2B4, but also of the associated LAT molecules. Furthermore, immunoprecipitation of 2B4 also

demonstrated the recruitment of PLCγ and Grb2. The association of 2B4 with LAT further extends the similarities of 2B4 signaling with TCR stimulation in addition to the requirement for co-stimulation for both receptors (Sivori et al., 2000).

SLAM is an activating receptor on human T cells and B cells. In T cells SLAM is involved in proliferation and IFN-γ production (Aversa et al., 1997b), and in promoting Th0/Th1 differentiation (Aversa et al., 1997a; Carballido et al., 1997). The SLAM cytoplasmic sequence of the long isoform contains two tyrosines embedded in the consensus motif. These tyrosine motifs in SLAM associate with the SH2 domain of SAP (Sayos et al., 1998), the defective gene product (SH2D1A, DSHP) in X-linked lymphoproliferative disease (XLPD) (Coffey et al., 1998; Nichols et al., 1998). Furthermore, 2B4 has been demonstrated to associate with SAP (Sayos et al., 2000; Tangye et al., 1999).

The SLAM associating protein SAP is a small cytosolic protein containing a single SH2 domain and a short tail. It is most similar to EAT-2 which is upregulated in Ewing's sarcoma (Thompson et al., 1996). The SH2 domains of both SAP and EAT-2 specifically recognize the sequence motif T-I-pY-x-x-(V/I), as determined by phosphopeptide library screening (Poy et al., 1999). Therefore the SAP / EAT-2 SH2 domain binding motif is the consensus tyrosine motif found in 2B4, SLAM, CD84, Ly9, and CS1.

Further intriguing data concerning the role of SAP is that it associates with p62(dok),a novel protein with features of a signaling molecule. The tyrosine phosphorylation of p62(dok) leads to its association with the p120 ras GTPase-activating

protein (GAP) (Yamanashi and Baltimore, 1997) which is required for inhibitory signaling through the FcγRIIB in B cells (Tamir et al., 2000). Furthermore, p62(dok) is rapidly tyrosine-phosphorylated upon activation of the c-Kit receptor (Panzenbock et al., 1998) and constitutively tyrosine-phosphorylated in hematopoietic progenitors from chronic myelogenous leukemia (CML) patients (Carpino et al., 1997). Therefore, phosphorylation and subsequent protein interactions of p62(dok) are probably tightly regulated. This could be mediated through reported associations with SHIP1 (Dunant et al., 2000), lck (Nemorin and Duplay, 2000) and SAP (Sylla et al., 2000). Mutant SAP molecules from XLP patients fail to associate with p62(dok). Furthermore, overexpession of SAP, regardless of mutation, activated NF-kB (Sylla et al., 2000). Perhaps dysregulation of the phosphorylation of p62(dok) and its lack of binding to GAP may also contribute to the lymphoproliferation seen in XLP. The presence of unique tyrosine motifs in the cytoplasmic domain of CS1, that might associate with SAP, further demonstrate that the immune dysregulation in XLP is complex and may involve signaling via CS1 in addition to 2B4 and SLAM.

CD2 subset members have been directly implicated in both immune regulation and disease. The anti-2B4 mAb, C1.7 did not stain $CD4^+$ T cells from normal human subjects, indicating that these $CD4^+$ T cells do not express 2B4. However, high avidity and donor specific $CD4^+$ CTLs observed in clinical heart rejection are $C1.7^+$ (van Emmerik et al., 1998). Furthermore, C1.7 expression on $CD8^+$ T cells in HIV patients is activation dependent (Peritt et al., 1999). This suggests that the expression of 2B4 correlates with activated or memory cytotoxic T lymphocytes.

SLAM also appears to play a role in HIV infection. Early HIV infection results in a reduction of SLAM expression which correlates with a time-limited impairment of cell-mediated immunity. Furthermore, triggering via SLAM potentiates HIV-specific proliferative responses with simultaneous down regulation of IL-10 and redirection of the response to TH0/TH1 by IFN-γ production.

CD48 is a GPI-linked surface molecule widely expressed on cells of hematopoietic origin and the high affinity ligand for 2B4. Cell surface expression of CD48 is upregulated in viral infections and by both alpha/beta and gamma interferon (Klaman and Thorley-Lawson, 1995; Tissot et al., 1997). Importantly, CD48 expression on B cells is upregulated by Epstein Barr virus (EBV) infection (Yokoyama et al., 1991). Soluble CD48 is detected at elevated levels in the plasma of patients with lymphoproliferative disease, arthritis, and acute EBV infection (Smith et al., 1997). Soluble CD48 may be the result of 2B4 – CD48 signaling as the GPI-linkage of CD48 may be cleaved. Alternatively, soluble CD48 may play an immune modulatory role by stimulating the 2B4 receptor.

XLPD was originally described by Purtilo and colleagues (Purtilo et al., 1975; Purtilo et al., 1977) as an X-linked recessive progressive combined variable immunodeficiency, with selective susceptibility to EBV infections. Since the original report in 1975, over 250 cases have been documented in the XLPD registry established by Purtilo. The most common maladies associated with XLPD are fulminant infectious mononucleosis, variable immunodeficiency, and malignant lymphoma. Usually, but not always, these conditions are triggered by EBV infection (Sullivan et al., 1983).

EBV is a γ herpes virus. In most of the world, primary infection usually occurs by the third birthday and is asymptomatic. However, when infection is delayed, infectious mononucleosis is more likely to occur. Transmission is by the oral route (saliva). EBV infects the oropharyngeal epithelium, which is the site of most EBV viral replication and where persistent infection is established (Khanna, Burrows and Moss, 1995). EBV has a strong tropism for B cells and latent infection is life long. Infection of lymphocytes occurs via interaction of CD21 with the major viral envelope glycoprotein gp305 (Birkenbach et al., 1993).

During infectious mononucleosis, the early immune responses include increased cytokine production, $CD4^+$ T cell and NK cell activation, and the generation of IgM antibodies to EBV proteins. Then, a large expansion of activated antigen specific $CD8^+$ CTLs is generated (Hoshino et al., 1999; Kuzushima et al., 1999). At the height of infectious mononucleosis, symptoms include lymphoadenopathy, pharyngitis, splenomegaly, and hepatic dysfunction. In immunocompetent patients this acute phase of EBV infection resolves. CTL responses diminish and a life long B cell latent infection is established. In XLPD patients who develop the fulminant infectious mononucleosis phenotype, the acute phase does not resolve. The uncontrolled immune response (referred to as virus associated hemophagocytic syndrome, VAHS) causes liver damage and bone marrow failure and is frequently fatal. The occurrence of VAHS places XLPD among the inherited hemophagocytic lymphohistiocytosis (HLH) syndromes, a family of disorders with both immune deficiency and uncontrolled activation of the cellular immune systems. In addition to XLPD, the inherited HLH disorders include familial hemophagocytic

lymphohistiocytosis (FHL), Chediak-Higashi syndrome, and Griscelli Syndrome (Dufourcq-Lagelouse et al., 1999). Defective lymphocyte cytolytic function is observed in these three HLH syndromes (Menasche et al., 2000; Nakazawa et al., 1999; Stepp et al., 1999a).

Maintenance of EBV in the latent phase requires the sustained production of virus specific IgG antibodies and memory CTLs. In vitro EBV infection immortalizes B cells. In immunosuppressed patients EBV driven lymphoproliferative disorders and malignancies may develop. EBV specific CTLs are absolutely critical in preventing and/or controlling EBV associated lymphoproliferative disorders and malignancies, and can be used in the treatment of these disorders in immunosuppressed patients (Heslop and Rooney, 1997; Khanna et al., 1999; Kuzushima et al., 1996; Rooney et al., 1998).

The expression of CD48, the 2B4 ligand, on B cells is upregulated by EBV infection (Klaman and Thorley-Lawson, 1995; Yokoyama et al., 1991). This suggests that EBV infected B cells may be targets for 2B4 mediated killing or cytokine production by 2B4 expressing NK cells and/or $CD8^+$ T cells, cells thought to be critical to the immune response to EBV. Furthermore, 2B4 associates with SAP in a tyrosine phosphorylation dependent manner (Sayos et al., 2000; Tangye et al., 1999). Finally, 2B4 mediated NK cell cytotoxicity is defective in XLPD patients (Benoit et al., 2000; Nakajima et al., 2000; Parolini et al., 2000; Tangye et al., 2000). Therefore, SAP dependent 2B4 function may be critical in the control of EBV infection and defective 2B4 function in the absence of SAP may contribute to XLPD including VAHS during acute EBV infection and the high incidence of B cell lymphomas.

In conclusion, it is apparent that NK cells play a major role in immune regulation and disease. Elucidation of the NK cell regulation requires identification and characterization of all receptors involved. Despite the fact that many NK cell receptors have been previously cloned, it is evident that they do not represent the full spectrum. I have isolated NK cell receptors from both the C-type lectin and Ig superfamilies of receptors. What role the LLT receptors play remains to be addressed. It is now evident that 2B4 plays a major role in the function of NK cells and other leukocytes. 2B4 was originally identified as a mouse NK cell receptor associated with non-MHC-restricted killing. Identification and functional characterization of human 2B4 has resulted in an explosion of the role of human 2B4 in the immune system in health and disease. Moreover, the identification of CD48 as the high affinity ligand for 2B4 in the light of previously obtained data on CD2 and CD48 knockout mice has implicated a role for 2B4 in the development of functional $CD4^+$ T cells. It is well accepted that NK cells are inactivated upon interaction with cells displaying self class I molecules. 2B4-CD48 interaction provides a MHC independent NK cell activation signal. The finding that a functional SAP is required for 2B4 activation of NK cells as revealed by XLPD, suggest that 2B4 mediated killing or 2B4 mediated cytokine release is critical in the control of EBV infections and defective 2B4 function may contribute to XLPD. Although several of the 2B4 associated molecules are known, the exact signaling pathway of 2B4 activation is still not understood. Furthermore, the function of 2B4 on other leukocytes remains unclear. The *in vivo* role of 2B4 in the immune system will be clearly demonstrated by studies in 2B4 knockout mice. The biological function as well as the nature of the ligand

of CS1 is not known. Structural features suggest that CS1 may have a similar role as that of 2B4. Further progress in the molecular and functional characterization of these two novel members of the CD2 subset will provide a better understanding of their role in the immune system.

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